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LOW PHOSPHOLIPID VALUES IN DOG PLASMA

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The oxidative method for the determination of blood lipids described by Bloor (5) was in 1929 (6) extended by him to the determination of phospholipids. Subsequently it was found in this laboratory (10) during the analysis of plasma phospholipids in a series of normal dogs under controlled living conditions and on a low fat diet that abnormally low, erratic, and irreproducible values were obtained with the technique as outlined. The present paper is concerned with a report on the plasma phospholipids of these dogs and with the modification in the method necessary for the determination of phospholipids at these very low levels.

Regarding the equation for the oxidation of lecithin, Bloor (1929) has calculated the cc. of 0.1 N $K_2Cr_2O_7$ required per mg. on the assumption that the nitrogen present goes to gaseous nitrogen. On the assumption that this process would occur through the stage of ammonia, producing $(NH_4)_2SO_4$, it was found by experiment that $(NH_4)_2SO_4$ was not oxidized. On this basis and correcting for a slip in balancing the equation in Bloor's paper, 1 mg. of lecithin requires 3.03 cc. of 0.1 N $K_2Cr_2O_7$. The equation is

$$2C_{42}H_{84}O_9NP + 118O_2 + H_2SO_4 \rightarrow 84CO_2 + 78H_2O + 2H_3PO_4 + (NH_4)_2SO_4$$

Similarly the figure for cephalin may be calculated to be 3.04 cc. With these factors in place of 3.11 and 3.12 the per cent recovery as based on Bloor's (6) figures for pure lecithin and cephalin is 99.6 and 96.4 per cent respectively. The practical factor of 3.00 cc. as used by Bloor is thus shown to be very near the theoretical on the basis that $(NH_4)_2SO_4$ is the end-product. However, the possi-

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bility of the production of other nitrogen derivatives such as amines (*e.g.* trimethylamine) has not been considered.

In this investigation phospholipid determinations were performed on healthy male dogs of approximately the same age and weight and under controlled conditions of temperature and exercise. The diet was essentially a low fat diet consisting of biscuits of the composition: crude fiber 2.14, fat 2.36, ash 8.60, protein 17.91, and carbohydrate 61.81. Blood was taken and plasma extracts were prepared 15 to 24 hours after a meal.

Method

The method of determination used is as follows: Blood samples of 50 to 60 cc. volume are withdrawn from the jugular vein into a flask previously rinsed with a saturated solution of sodium citrate, and immediately centrifuged. For plasma determinations exactly 25 cc. are pipetted off and run into about 400 cc. of alcohol-ether (3:1, both redistilled) in a 500 cc. volumetric flask, the plasma being allowed to run down the side of the flask which is continuously rotated. After standing with occasional shaking for 24 hours or longer the mixture is made up to volume and quickly filtered through an alcohol-extracted filter paper.

100 cc. samples of the alcohol-ether extract are evaporated to dryness on the steam bath and extracted with redistilled ethyl ether four times, bringing the solvent to a gentle boil each time, so as to make a final volume of about 10 cc. of ether solution per sample. These are then centrifuged, the clear liquid transferred quantitatively to 15 cc. graduated centrifuge tubes, concentrated to 2 cc., and treated with 7 cc. of dry redistilled acetone and 3 drops of a saturated alcoholic solution of $MgCl_2$ as in Bloor's procedure. One-half hour is allowed for acetone precipitation after which the tubes are centrifuged for 7 minutes at 1500 revolutions per minute, the acetone-ether poured off, and the precipitate washed twice with 2 cc. portions of acetone, breaking up the precipitate with a clean glass rod and centrifuging each time. After the last washing, the precipitates are drained, dried with a current of air, and dissolved in 5 cc. of moist ether (peroxide-free), with a clean glass rod drawn out to a fine point to dislodge the precipitate, and rotating the tubes to encourage solution. The tubes are then centrifuged and the dissolved material transferred to 125 cc. glass-

stoppered Erlenmeyer flasks as described by Bloor. The moist ether solution is evaporated to dryness on the steam bath, the residue treated with 5 cc. of silver reagent (silver dichromate, see Bloor (6)) and exactly 3 cc. of 1 N $K_2Cr_2O_7$, and let stand 20 to 30 minutes so that all flasks will be at the same temperature on entering the oven and hence be uniformly heated in the next step of the procedure. This consists in completing the oxidation by heating in an electric oven at 124 $\pm 2°$ for 15 to 20 minutes keeping the flasks loosely stoppered for the first 7 minutes and closed for the remainder of the time. The flasks are removed from the oven, the stoppers loosened, and 75 cc. of ice-cold distilled water quickly added to each. They are then transferred quantitatively to 300 cc. flasks, diluted to a volume of 200 cc. with distilled water, and titrated with 0.1 N $Na_2S_2O_3$ after the addition of 10 cc. of 10 per cent KI and with a 1 per cent starch solution as an indicator. One analysis usually consisted of four samples and two controls with all the reagents except the lipid substance.

The true normality of the thiosulfate is determined and the titration value of the phospholipid, obtained by subtracting from the mean of the blanks, expressed as cc. of 0.1 N $K_2Cr_2O_7$. The mg. of phospholipid present may then be calculated by dividing this figure by the factor 3.00 (3.00 cc. of $K_2Cr_2O_7$ = 1 mg. of phospholipid). Multiplying the value thus obtained by 20, *i.e.* $\frac{100}{5}$, gives the amount of phospholipid present in the original plasma as mg. per cent by volume.

The essential differences between the above procedure and that proposed by Bloor (1929) are (1) the alcohol-ether extract is not brought to boiling, (2) a larger aliquot of alcohol-ether extract is needed, (3) ethyl ether instead of petroleum ether is used to extract the alcohol-ether residue, (4) the acetone precipitate is washed more thoroughly, (5) the titration mixture is diluted to 200 cc., and (6) two blanks instead of one are used. It was found by trial that essentially the same phospholipid values were obtained with or without heat in the extraction of these plasmas with alcohol-ether. The reasons for the complete extraction without heat are probably the large volume of solvent relative to plasma (19 to 1), the time of extraction (in most cases the extracts stood 3 to 4 days before being made up to volume and filtering), and thirdly the

fact that in heating a larger portion of the better solvent, ether, will be lost.

The use of larger amounts of blood and of alcohol-ether extract and therefore of phospholipid was found to be the key-note for obtaining consistent and reproducible results with these plasmas of low phospholipid content. The procedure was suggested by the results obtained in determining the percentage recovery of lecithin in varying concentrations with and without the presence of plasma.

Table I exhibits the recovery of known amounts of lecithin as determined by this method and it may be seen that the loss of lecithin relative to the amount used becomes very appreciable below about 2 mg. initially present. When known amounts of lecithin were added to a plasma extract and the recovery deter-

TABLE I

Percentage Recovery of Varying Concentrations of Lecithin

Lecithin present	Lecithin recovered	Recovery	Standard deviation in recovery
mg.	*mg.*	*per cent*	
3.57	3.72	104	1.7
1.48	1.90	107	12.3
0.713	0.295	41.4	23.8
0.357	0.146	40.9	12.9

mined, similar low percentage values were obtained with less than 2 mg. of added lecithin.

On a series of eighteen blank determinations without phospholipid it was found that two determinations might vary by as great as 0.4 cc. in titration value. The standard deviation, however, was 0.16 cc. Hence it is obvious that where only one blank is used the determined phospholipid value may vary by as much as 0.1 to 0.2 mg. with an expected variation of 0.05 mg. due to errors in the blank. Such a variation becomes increasingly significant with smaller amounts of determined phospholipid and since it will always be a source of experimental error it was considered advisable to minimize the error by the introduction of two blanks.

With these considerations applied to plasma extracts, in Table II are given the determined values of phospholipid with varying amounts of the same alcohol-ether extract. Each figure is the

mean of three determinations, the standard deviations being indicated. It will be apparent that in estimating less than 2 mg. of phospholipid, higher and more variable values are obtained than with larger amounts. With 20 cc. of alcohol-ether extract the variations were even greater than those given in Table II for 50 and 100 cc. Repeated analyses with 20 cc. gave figures for these plasmas which ranged from 60 to 170 mg. per cent with an average standard deviation for each determination of 10 mg. per cent. It is obvious, therefore, that less than 2 mg. of phospholipid cannot safely be determined by this method. 100 cc. volumes of alcohol-ether extract were found with these plasmas to give a thiosulfate

TABLE II

Relation of the Determined Phospholipid Value to the Amount of Phospholipid Present in Blood Plasma

Dog No.	Determined phospholipid level of blood plasma					
	With 50 cc. alcohol-ether extract			With 100 cc. alcohol-ether extract		
	Phospholipid found	Phospholipid	Standard deviation	Phospholipid found	Phospholipid	Standard deviation
	mg.	*mg. per cent*		*mg.*	*mg. per cent*	
29-209	1.41	56	2.5	2.55	51	1.6
29-210	1.91	76	8.4	3.05	61	1.4
29-199	1.60	64	1.0	2.88	58	1.5
29-288	1.88	75	4.2	3.05	61	0.8
Mean...	1.70	68	4.0	2.88	58	1.3

titration difference representing about 9 cc. of 0.1 N $K_2Cr_2O_7$, indicating the presence of about 3 mg. of phospholipid, and the results, as judged by standard deviations in triplicate and repeated determinations, were very satisfactory.

Ether has been substituted for petroleum ether in extracting the alcohol-ether residue because it was found to give more consistent and slightly higher phospholipid values. While ether is a better lipid solvent than petroleum ether, it dissolves some non-fat substances. The latter objection was met by introducing two thorough acetone washings and scrubbings.

Precipitation of phospholipids from plasma extracts has two main objections: from a quantitative point of view, the precipitate

Low Phospholipid Values in Plasma

is contaminated with impurities present in the solution and secondly phospholipids are soluble in acetone to a slight extent in the presence of fat or fatty acids (9, 13). Determinations of known amounts of lecithin in the presence of varying concentra-

TABLE III

Plasma Phospholipid Levels of Dogs on Low Fat Diet

Dog No.	Type	Weight	Date	Mean phospholipid value	Standard deviation	Mean phospholipid value per dog	Standard deviation per dog
		kg.		*mg. per 100 cc.*			
29-209	Pointer	24.8	Mar. 19	54	1.0		
		24.8	" 21	43	0.8	49	4.6
		20.4	Aug. 12	51	1.6		
29-199	Coach	24.4	Mar. 14	43	0.0		
		24.4	" 20	47	0.57	49	5.5
		24.5	Aug. 15	58	1.5		
29-210	Pointer	20.7	Mar. 12	58	3.1		
		20.7	" 19	50	3.3	51	10.5
		20.7	" 21	34	1.0		
		19.1	Aug. 15	61	1.4		
29-288	Police	19.0	Mar. 12	40	1.0	51	10.6
		19.5	Aug. 14	61	0.8		
29-203	Coach	25.6	Mar. 18	48	4.5	52	3.5
		25.6	" 21	55	1.4		
29-225	Collie	20.0	Mar. 4	64	5.3	59	5.0
		20.0	" 19	54	2.1		
29-207	Coach	20.8	Mar. 11	71	2.5	62	9.0
		20.8	" 21	53	1.0		
29-196	Coach	19.7	Mar. 14	76	11.0	76	
29-228	Collie	19.0	Mar. 13	134	2.1	111	23.0
			" 20	88	4.9		
Mean (without Dog 29-228).......				53.7±9.4θ	2.3		7.0

tions of oleic acid indicated that the first of these was relatively the more important in this case as recoveries increased to well over 100 per cent. This plus the fact that better duplicates were obtained seems to justify the value of the extra acetone washing.

Results

In Table III are tabulated the results of a series of analyses on nine dogs on a low fat diet over a period of 5 months. Each phospholipid value represents the mean of at least three determinations. The standard deviations have been calculated from the formula

$$\vartheta = \sqrt{\frac{\Sigma (x)^2}{n}}$$

where x stands for the deviation of each value from the mean and n the number of readings (8). The earlier extracts were prepared by Glusker in this laboratory and were analyzed later when the above method for their determination had been evolved. Excluding Dog 29-228, which, as shown by the drop in phospholipid value in 1 week, had obviously not attained the low equilibrium values characteristic of the other eight when samples of blood were removed for analysis, the mean value for all dogs was 53.7 mg. per 100 cc. of plasma with a standard deviation of 9.4 mg. The mean sigma per dog over this period was 7.0 mg. per 100 cc. plasma.

The phospholipid values of the blood corpuscles were also determined in a series of four dogs on the same diet and the figures obtained are shown in Table IV. Each mean is the average of four determinations and the results are reported as mg. per 100 gm. of cells (mg. per cent). The white cells were obtained by skimming off into a beaker the leucocyte layer on the surface of the red cells after centrifuging the blood. The beaker and white cells were then weighed, the whites quickly poured into about 75 cc. of alcohol-ether in a 100 cc. volumetric flask, and the weight of the beaker again found. Usually about 5 gm. of white cells were obtained from 250 cc. of blood by this procedure. Some red cells and plasma were necessarily included and these, together with the fact that results are reported as per cent wet weight, rather than dry weight, tend to bring the determined phospholipid values lower than those actually present. The red cells were similarly obtained by weighing about 5 gm. of the red cell layer thoroughly mixed after removal of the plasma and white cells. 10 cc. of alcohol-ether extract proved a convenient volume for analysis; otherwise the procedure was the same as that described above.

The mean value for erythrocytes was found to be 372 mg. per

cent and was fairly constant having a standard deviation of only 9.3 mg. The phospholipid content of the white cells was much higher, almost twice that of the red cells, showing a mean value of 710 mg. per cent. In the latter case the standard deviation was much greater than with the red cells, 86 mg., but this is undoubtedly a reflection of the variations in the method of obtaining white cells. The mean standard deviations per phospholipid determination were higher than in the case of plasma, Table III. However the means were different. If, for comparative purposes, the per cent standard deviations, *i.e.* sigma per 100 mg., are calculated, then the following values are obtained: plasma, 4.3 per cent; red cells, 3.0 per cent; and white cells, 1.9 per cent. The mean amounts of phospholipid determined in each case were:

TABLE IV

Phospholipid Content of Blood Corpuscles in Dogs on Low Fat Diet

Dog No.	Red cells		White cells	
	Mean	θ	Mean	θ
	mg. per 100 gm.		*mg. per 100 gm.*	
29-288	388	11.0	595	13.0
29-199	367	9.8	668	1.8
29-209	364	15.0	754	14.0
29-210	370	8.6	821	25.0
Mean.......	372±9.3θ	11.1	710±86θ	13.5

plasma, 2.67 mg.; red cells, 2.96 mg.; white cells, 4.26 mg. Hence it would appear that the standard deviation varies inversely with the amount of phospholipid analyzed.

Before mixing the layer of red cells in one experiment samples were removed from the top and bottom of this layer. Identical phospholipid values were found in both. Hence it may be concluded that the high phospholipid content of the white cells was not due to their position at the surface of the red cell layer.

DISCUSSION

Bloor (5) emphasized the necessity of not using over a certain maximum amount of fatty acid, which would correspond to about 4 mg. of phospholipid, in the oxidation procedure with 3 and 5 cc.

of dichromate and silver reagent respectively; *i.e.*, the oxidation mixture after heating should be definitely brown and not greenish. In the present work it has been shown that there is also a minimum of phospholipid, 2 mg., below which satisfactory results are impossible. Hence with the amounts of reagents specified the range of analysis by this micro procedure is 2 to 4 mg. of phospholipid; larger amounts, however, may be determined by using multiples of the oxidizing reagents. With experience the method is quite satisfactory giving values which vary by a per cent standard deviation of less than 5 per cent with 2 mg. of material and 2 per cent with 4 mg.

It has been shown that under controlled conditions dog plasma phospholipid remains at a constant level with a standard deviation of 17.5 per cent from the mean of all dogs for a period of 5 months. This is in accordance with the findings of Glusker (10) that under similar conditions the cholesterol level shows a standard deviation of 28 per cent and a total fatty acid 13 per cent from the average of the means. It may thus be concluded that under constant conditions dog plasma lipid levels are maintained at constant values.

The second feature of the plasma phospholipid levels here reported is their low values, 49 to 76 mg. per cent. With the nephelometric method previous values found by Bloor (1) for dog plasma were 330 mg. per cent and 240 mg. per cent (4), in "normal young dogs" 254 to 478 mg. per cent (7). After a 24 hour fast four dogs examined by Bloor (2) were found to have plasma phospholipids varying from 310 to 370 mg. per cent, the value rising 30 to 90 mg. after the ingestion of a high fat meal. The rise began in about 2 hours, reached a peak at 5 hours, and had not returned to the previous fasting level after 8 hours. Similar results were obtained by Bloor, Gillette, and James in their control dogs (7). The postprandial rise in plasma phospholipid was not, however, always obtained and in several instances was quite small. Furthermore, Horiuchi (11) found that rabbits had approximately the same plasma phospholipid values on a low or high fat diet. From the evidence available in the above work, therefore, it is impossible to ascribe the low phospholipid values here found to the low fat of the diet.

In addition to being low in fat, this diet was high in carbohydrate.

In this connection it is interesting to note that animals whose diet is essentially high in carbohydrate, such as rabbits, cows, sheep, etc., have lower plasma phospholipid values than the carnivorous animals, dogs, cats, etc. (1, 4, 11). Human plasma comes about intermediate in the scale (4, 12).

That these low values represent the true phospholipid levels of dogs on a low fat-high carbohydrate diet seems fairly certain. In the first place, due to individual peculiarities in the dogs studied such low values would not be expected to show up in eight dogs of various species unless rather a common occurrence, and if so,

TABLE V

Relation between Phospholipid and Cholesterol Levels in Plasma of Dogs on Low Fat Diet

Dog No.....	29-288	29-199	29-210	29-209	29-203	29-225	29-207	29-196	29-228
Phospho-lipid, *mg.* per 100 cc...	40	45	47	49	52	59	62	76	111
Cholesterol, *mg. per 100* cc..........	68	108	124	98	115	93	138	139	188
Fatty acid, *mg. per 100* cc..........	212	243	240	243	297	243	302	348	348
$\dfrac{\text{Phospholipid}}{\text{Cholesterol}}$	0.58	0.42	0.38	0.50	0.45	0.63	0.45	0.56	0.59
$\dfrac{\text{Fatty acid}}{\text{Phospholipid}}$	5.3	5.4	5.1	5.0	5.7	4.1	4.9	4.6	3.1

it is likely to have been noticed before. The possibility that the method does not determine the total amount of phospholipid present was ruled out by experiment where it was found that 80 to 90 per cent of a known amount of lecithin added to plasma extract could be recovered. However, it may be that there is present in these plasmas some form of phospholipid not determined by the ordinary methods for isolation.

From data available through the work of Glusker (10) on the cholesterol and fatty acid content of the same extracts of these plasmas, the phospholipid:cholesterol and fatty acid:phos-

pholipid ratios have been calculated and are shown in Table V. It may be seen that under these controlled environmental and dietary conditions fairly constant values are obtained. Similar findings have been reported by other investigators (3, 11, 14) but it is impossible to compare the values for these ratios given in Table V to the "normal" value because no normal values exist. For, as shown by Mayer and Schaeffer (15) the ratios are altered by environmental conditions.

While the phospholipid content of the blood plasma is low, that of the corpuscles, *i.e.* 372 mg. per cent for the red cells, is very similar to other reported values. Thus Bloor (4) gives the value 440 mg. per cent for dog corpuscles. In this connection it is further interesting to note that there is a relatively slight variation in the corpuscle content of phospholipid in herbivorous and carnivorous animals and man (4, 11, 12). The high phospholipid content of the white cells may be significant in view of the recent findings of Larson (16) that lipids function in the neutralization of bacterial toxins.

SUMMARY

Modifications have been devised for the application of Bloor's oxidative method to the determination of low (50 mg. per cent) phospholipid values in dog plasma. It has been shown that this method cannot be used with safety for the estimation of less than 2 mg. of phospholipid but that it is entirely satisfactory with amounts of this order or greater.

Eight dogs under controlled living conditions and on a low fat-high carbohydrate diet were found to have a mean plasma phospholipid content of 53.7 mg. per cent with a standard deviation of 9.4 mg. The significance of these low values has been discussed and their constancy compared with that of the other lipids in plasma. Phospholipid values for erythrocytes and leucocytes have also been determined by this method, discussed, and compared with other values in the literature.

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THE INABILITY OF METALS OTHER THAN COPPER TO SUPPLEMENT IRON IN CURING THE NUTRITIONAL ANEMIA OF RATS

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The question of the effect of certain heavy metals upon blood regeneration in nutritional anemia is controversial in nature. The literature on the subject is too well known to demand an extensive review. Waddell, Steenbock, and Hart (1) maintain that copper as a supplement to iron is unique in its action on hemoglobin regeneration in a condition of nutritional anemia produced in albino rats by a diet of whole milk only; whereas Titus, Cave, and Hughes (2) have obtained results which indicate that manganese is also effective and that the manganese-copper-iron complex is most active in hemoglobin regeneration. Moreover, Myers and Beard (3) have found that a number of metals, supplementing iron, have a marked erythropoietic action. On account of the discrepancy in results, it seemed advisable to repeat part of this work in another laboratory.

EXPERIMENTAL

With a few exceptions the procedure followed was that of Waddell, Steenbock, Elvehjem, and Hart (4). Male albino rats, 28 days old and weighing from 40 to 60 gm., were taken at the time of weaning, placed in glass cages, and fed on a diet of liquid whole cow's milk. At the end of 8 days, the animals were placed in individual glass cages and the weights were determined weekly. After 25 days, weekly hemoglobin determinations were made by the Newcomer hemoglobinometer. Blood was obtained by clipping the tail. When the hemoglobin level reached 3 to 4 gm. per 100 cc. of blood, the supplements to the milk diet were given daily (including Sundays).

Cages

A glass cage (Fig. 1) was designed[1] so that the animals could not come in contact with anything except glass, yet could have the ventilation and exercise afforded by wire cages. A rectangular base for the cage was made by fastening together two soft pine strips, $1 \times 2 \times 30\frac{1}{2}$ inches with pieces of $1\frac{1}{2}$ inch lathing $9\frac{1}{2}$ inches long in such a way as to form a rigid frame. This was accomplished by nailing to the side strips at each end two pieces of the lathing, one across the ends with its narrow edge even with the tops of the
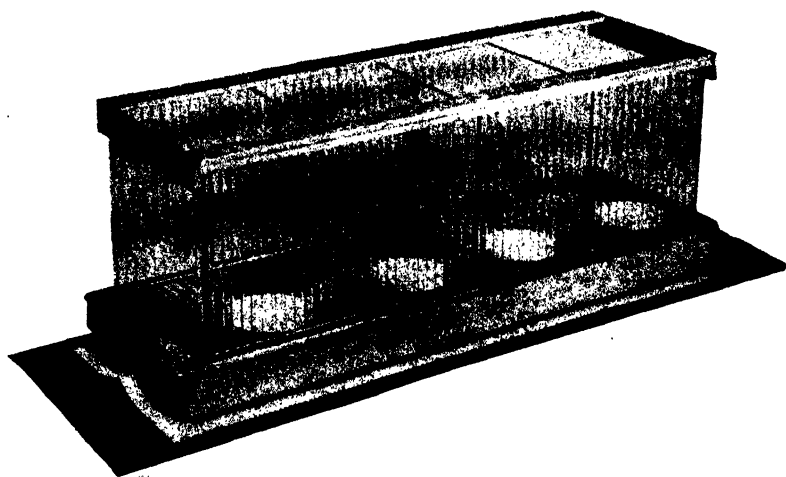
Fig. 1. Glass cage designed for use in the study of metals in nutritional anemia.

side strips and the other on top at the extreme ends. For supports, four short strips $\frac{1}{4}$ inch thick were tacked to the bottom of the base at the corners. The sides of the cage were made by mounting firmly $10\frac{7}{8}$ inch lengths of 5 mm. glass tubing in holes bored by means of a drill press in the sides of the base to a depth of $1\frac{1}{2}$ inches with centers $\frac{1}{2}$ inch apart.[2] The floor consisted of 9 inch lengths

[1] This cage was in use prior to the description of a different type of glass cage by Nevins, W. B., and Shaw, D. D., *Science*, **72**, 249 (1930).

[2] A small amount of vaseline placed in the holes facilitated the mounting of the pieces of tubing.

of 7 mm. tubing laid between the upright tubing and held in place by ¼ inch strips of wood tacked to the top outer edge of the sides of the base. These pieces of tubing were held down by two lengths of 3 mm. glass rodding running the length of the floor, on either side, just inside the upright tubing. These rods also prevented the animals from gaining access to the wooden base. Panes of window glass 9 inches square formed the ends of the cage and the partitions between the compartments. These glass plates could be slipped between two upright tubes at any desired point, thus enabling one to adjust the number of compartments from one to six. Cages of four compartments, each approximately 9 × 7 inches, were found most satisfactory. Since the glass partitions rested on the long pieces of glass rodding which were used to hold the floor in place, a 3 mm. space was left between the bottom of each partition and the floor tubing. In order to insure complete separation of the compartments, the gap was closed by means of a 7¾ inch length of 7 mm. glass tubing held in place by a 9½ inch length of 3 mm. glass rodding which extended through the tubing and into the space between the partition and the nearest upright tube at either side of the cage. The top was made of 9 × 28 inch window glass set in a light wooden frame. This glass rested on the partition and end plates which extended ¼ inch above the upright glass tubing of the sides. The top fitted firmly, but could be easily removed when it was necessary to open the cage. The completed cage was placed on a glass plate covered with paper toweling to receive the excreta. Toweling was used to avoid the possibility of the introduction of traces of metals from sawdust or shavings which, if used, might adhere to the tails of the rats.

This type of glass cage is inexpensive, easily made from common laboratory materials, and provides the animals with quarters equal to those offered by the various wire cages ordinarily used for experimental studies on. rats. Furthermore, the construction of the cage prevents the experimental animals from coming in contact with any metal whatsoever and thereby avoids the possibility of the introduction of extraneous metals derived from the cage. The use of such a cage for study of the effects of metals in nutritional anemia is, we believe, of fundamental importance.

Milk

All milk was collected directly into a glass jar having a wide mouth and a ground glass stopper. Before each collection of milk, the jar was washed with soap and water, rinsed with tap water, then with distilled water, and finally with water redistilled from glass. To avoid any possible contamination due to carelessness, each milking was personally supervised by one of us. The milk was transferred to the feeding dishes by means of a pipette.

Feeding Dishes

Halves of Petri dishes were used as food containers, because they were easily cleaned and there was very little possibility of absorption of the added salts by the pores of the dish.

Preparation of Salts

The effects of iron alone and of iron supplemented by copper, manganese, cobalt, nickel, and zinc, respectively, were studied. In the preparation of the salts of these metals, water redistilled from glass and Schleicher and Schüll No. 590 filter paper were used throughout.

Iron—Ferric chloride was prepared by dissolving Baker and Adamson's iron wire for standardization in 1:1 hydrochloric acid. The resulting ferrous chloride was crystallized, dissolved in water, and saturated with hydrogen sulfide under pressure and with constant shaking for 24 hours. The solution was filtered twice and the filtrate was concentrated and oxidized with concentrated nitric acid. The nitric acid was removed by heating with an excess of concentrated hydrochloric acid. The resulting strong hydrochloric acid solution was slowly evaporated on a water bath, while being constantly saturated with hydrogen chloride gas. When crystals began to form, the concentrated solution was placed in a desiccator for complete crystallization. After large crystals had formed, the mother liquor was decanted and the crystals were returned at once to the desiccator for several hours before weighing. The ferric chloride was fed at the accepted level of 0.5 mg. of iron daily.

Copper—Cupric sulfate (Mallinckrodt's c.p.) was used. This was recrystallized several times from redistilled water. The salt

was fed in an aqueous solution at levels of 0.05 mg. and 0.025 mg., respectively, of copper daily.

Manganese—Manganous sulfate was prepared by dissolving manganese sulfate (Baker's c.p.) in water, making the solution slightly acid with hydrochloric acid, and saturating with hydrogen sulfide under pressure and with shaking for 24 hours. The solution was filtered and the filtrate was concentrated and allowed to crystal-
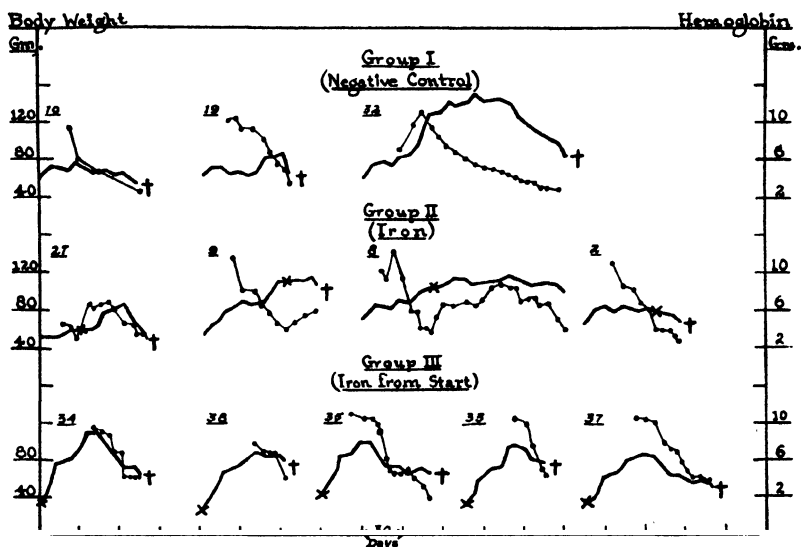
CHART I. Growth curves (heavy solid lines) and hemoglobin curves (light lines, with dots to indicate time of determinations) of male albino rats on basal diet (Group I), basal diet later supplemented by iron (Group II), basal diet supplemented by iron from start (Group III). The iron was fed at the level of 0.5 mg. daily in all cases. The cross on the curve of body weight marks the point at which iron was added to the basal diet. The dagger indicates death.

lize. The purified manganese sulfate was fed in an aqueous solution at levels of 5.0 mg. and 1.0 mg., respectively, of manganese daily.

Cobalt—Cobaltous chloride (Mallinckrodt's c.p.) was similarly prepared and fed at levels of 1.0 mg. and 0.5 mg., respectively, of cobalt daily.

Zinc—Zinc sulfate (Merck's c.p.) was used. This was prepared in the same manner. The solution was fed at levels of 1.0 mg. and 0.5 mg., respectively, of zinc daily.

Nickel—A solution of nickel sulfate was similarly prepared from nickelous sulfate (Baker's c.p.). This was fed at only one level of 1.0 mg. of nickel daily.

Results

In Group I (Chart I) are shown the weight and hemoglobin curves of control animals fed on a diet of liquid whole milk only throughout the entire experimental period. A pronounced nutritional anemia resulted in all cases. One animal, Rat 32, which was being kept in a cage 3 inches higher than that described, climbed the sides of the cage, spread the tubing at the top, and escaped[3] 3 days after the first hemoglobin determination was made. No food was present in the room; however, the animal was found near a copper-iron steam bath which *may* have furnished a source of metals and thus account for the sharp temporary rise in the hemoglobin and the subsequent increase in body weight.

Animals given iron supplements when the anemia had become pronounced (Group II, Chart I) showed a slight temporary rise in hemoglobin. The final results of this group, however, indicate that iron alone is ineffective in curing nutritional anemia in the albino rat.

In the experiments of Group III, a slightly different technique from that used in the other groups was followed. The animals were taken from their mothers at the age of 4 weeks, placed directly in individual glass cages, and fed the basal diet with the addition of 0.5 mg. of iron daily throughout the entire experiment. The experiments with this group of rats were started after it had been ascertained that iron alone was ineffective in curing the nutritional anemia produced in the animals of Group II. It was felt that iron might possibly be effective in preventing the development of anemia in animals that were in excellent nutritive condition, even though it had proved of no value as a curative agent when fed to sick (anemic) rats. As may be observed in Chart I, a severe nutritional anemia developed in the animals of this group,

[3] Cages with sides 9 inches in height, as the cage description specifies, have proved secure.

showing that the iron was just as ineffective in preventing the development of nutritional anemia as it was in curing it. The fall in hemoglobin was as rapid in the animals of this group, which received iron as a supplement to milk from the start of the experiment, as was found with those receiving milk alone.

CHART II. Growth curves (heavy solid lines) and hemoglobin curves (light lines, with dots to indicate time of determinations) of male albino rats on basal diet later supplemented by iron (0.5 mg. daily) and cobalt (Group IV), nickel (Group V), and zinc (Group VI), respectively. The figures in parentheses under the rat numbers indicate the amount of the metal other than iron fed daily. The cross on the curve of body weight marks the point at which the supplements of iron and other metal were added to the basal diet. The dagger indicates death.

In Chart II (Groups IV, V, VI) are shown the weight and hemo-
globin curves of rats which had iron with cobalt, nickel, and zinc,
respectively, added to the basal diet after the nutritional anemia
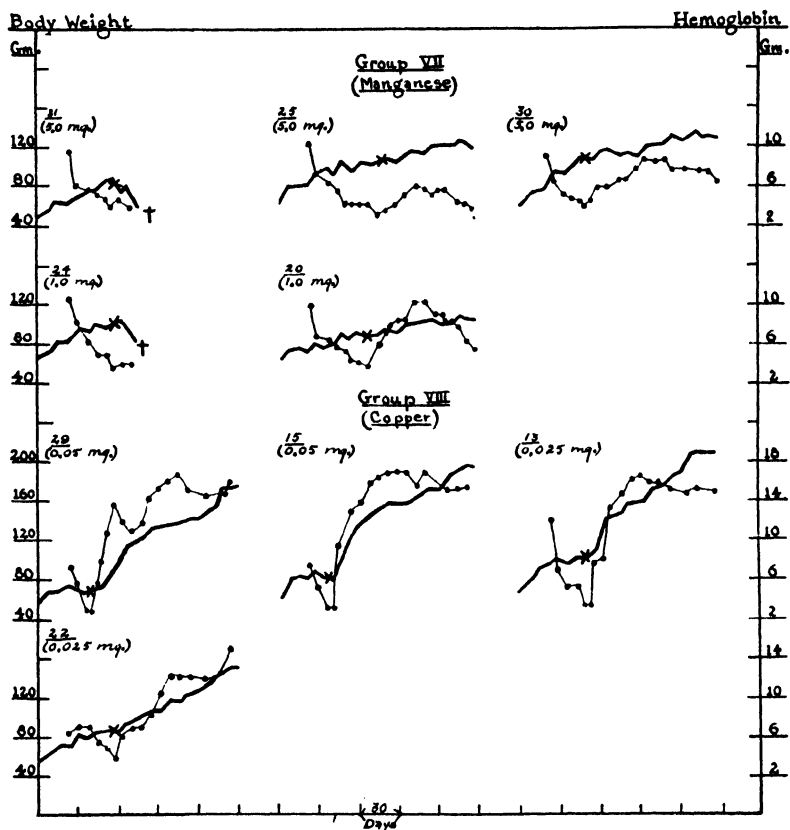
CHART III. Growth curves (heavy solid lines) and hemoglobin curves
(light lines, with dots to indicate time of determinations) of male albino rats
on basal diet later supplemented by iron (0.5 mg. daily) and manganese
(Group VII) and copper (Group VIII), respectively. The figures in paren-
theses under the rat numbers indicate the amount of the metal other than
iron fed daily. The cross on the curve of body weight marks the point at
which the supplements of iron and other metal were added to the basal diet.
The dagger indicates death.

had become pronounced.   As may be readily observed, the results
with these metals, fed at comparatively high levels, were uni-

formly negative. Although a slight transitory rise in hemoglobin occurred after the metals were added to the diet, all of the animals continued to lose weight, and the hemoglobin levels, which in no instance had risen above 7.1 gm., showed a subsequent fall except in the case of Rat 12 (Group IV) on cobalt and Rat 21 (Group V) on nickel. In the latter animal, the hemoglobin rose from 3.5 gm. at the time the nickel and iron were added to the diet to 7.1 gm. in 4 weeks and remained there unchanged for 25 days, the last hemoglobin determination at this level having been obtained 3 days before the death of the animal. This can hardly by considered recovery from a nutritional anemia, as a hemoglobin content of 7.1 gm. is no more than half of the generally accepted value for normal rats of this age.

Manganese was fed at the high levels of 1.0 mg. and 5.0 mg. daily (Group VII, Chart III). Two of the animals, Rats 24 and 31, died 13 and 19 days, respectively, after the supplemental additions were begun. The other animals showed the same slight temporary regeneration of hemoglobin as was found when iron alone, or with cobalt, nickel, or zinc, was added to the diet. Rat 20 attained a hemoglobin reading of 10 gm. in 5 weeks after the addition of the iron and manganese. This rise, however, lasted only a week, after which time a definite decrease occurred. It may also be noted that there was no definite increase in the weight of this rat, or of any of the other animals in this group, following the addition of the supplements. The slow gradual decrease of hemoglobin, exhibited by Rat 30 after the initial rise, may well be due to some factor other than manganese, since a similar response was observed in Rat 8 (Chart I) which received no addition of metal except iron. The results with this group of animals showed that manganese failed to produce a permanent marked regeneration of hemoglobin when added to an iron-milk diet.

When copper was fed at levels of 0.025 mg. and 0.05 mg., respectively (Group VIII, Chart III), there was in all cases a marked rise in hemoglobin to above 14 gm. per 100 cc. of blood. This was accompanied by a steady increase in weight, a response that did not occur in the animals of any other group. A striking improvement in physical condition resulted within 2 weeks after the addition of the supplements of copper and iron. The depression in the hemoglobin curve of Rat 29, which began 18 days after copper

was added to the diet and when the hemoglobin level had reached 14 gm., was probably caused by a spreading infection which developed in the tail. This was treated by cutting off the tail a little above the site of the infection and cauterizing the wound with phenol. The animal soon recovered and the hemoglobin rose again to a point even higher than that previously attained. The results with this group of rats show that small amounts of copper supplementing iron cause a marked regeneration of hemoglobin and a recovery from a condition of nutritional anemia produced by a diet of whole milk.

DISCUSSION

In general, the results of the experiments described warrant the conclusion that, of the metals studied, copper is the only one which is effective as a supplement to iron in curing nutritional anemia. One cannot conclude without qualification that no regeneration of hemoglobin occurred when metals other than copper constituted the supplement to iron, or when iron alone was fed. A number of the animals, given iron either alone or with cobalt, nickel, zinc, or manganese after a marked nutritional anemia had developed, showed a slight temporary increase in hemoglobin. With the exception of Rat 21, which has been discussed under the results of experiments with nickel (Group V), and Rats 9 (iron alone, Group II) and 12 (cobalt, Group IV), which died in 24 and 33 days, respectively, after the supplements of metal were added to the diet, a fall in hemoglobin has followed the initial rise. Furthermore, it should be noted that the same type of hemoglobin increase was found in the rats given no other metal than iron as occurred in those to whose diet other supplements were added. Consequently, we cannot conclude that cobalt, nickel, zinc, and manganese, when used separately as supplements to iron, are responsible for this temporary regeneration of hemoglobin.

Whether storage of copper, derived by the animals before the experimental period was begun, can explain the increase in hemoglobin which followed the addition of iron to the diet cannot be determined from our experimental data. That the rat is able to store copper when the diet contains this metallic element has been shown by a number of workers, including Lindow, Peterson, and Steenbock (5) and Flinn and Inouye (6). The possibility

of such storage being a factor in experiments on nutritional anemia has been suggested by Titus and Hughes (7). However, Drabkin and Waggoner (8) have reported that rats which have had copper in their ration are no more resistant to the onset of milk anemia than rats which have been on a low copper diet, a conclusion which is contrary to the experimental observations of Lindow, Peterson, and Steenbock (5). Certainly, more experimental evidence is necessary before it will be possible to decide whether the previous storage of copper plays any part in the results obtained in experimental studies on nutritional anemia produced when milk alone is fed to rats.

The reports of Myers and Beard (3), Mitchell and Schmidt (9), and Mitchell and Vaughn (10), that iron alone is effective in curing nutritional anemia produced in rats by a milk diet, are not supported by our experimental findings. These workers found definite regeneration of hemoglobin in all cases when iron, the only metal given, was fed in the form of ferric chloride at levels comparable to the 0.5 mg. quantity given daily by us. In no case did our animals (Group II) show more than a transitory increase in hemoglobin following the administration of iron after a definite anemia had developed. Our results with the animals of Group III, where iron was used as a supplement to the milk diet from the very beginning of the experiment, give further evidence that iron alone is ineffective in causing hemoglobin formation. The failure of iron to prevent nutritional anemia in the rats of this group confirms the observations of Titus and Hughes (7) that nutritional anemia is produced by a milk diet even when iron is present throughout the entire experimental period. In contrast to their results, however, the length of time required for the production of a severe anemia by the milk-iron diet was no greater than that required by a diet of whole milk only.

Inasmuch as the milk diet supplemented by iron alone was not capable of curing or preventing nutritional anemia in rats (Groups II and III), these experimental animals serve as excellent negative controls, for the results obtained with them show that the milk and the iron salt together did not contain sufficient copper to vitiate the results obtained when other metals were studied. Consequently, although it had been our intention to analyze the milk used for copper in case iron alone proved effective as a supplement

to milk in the correction of nutritional anemia, our negative findings led us to abandon this plan. Knowledge of the copper content of the milk would in no way affect the conclusions which may be drawn from our experiments.

Attention should be called to the comparatively high levels at which cobalt, nickel, zinc, and manganese were fed. Positive results with 0.1 mg. of manganese daily as a supplement to iron in the correction of nutritional anemia have been reported by Titus, Cave, and Hughes (2), and by Myers and Beard (3). Likewise the latter investigators have claimed that cobalt, nickel, and zinc, as well as a number of other metals, have erythropoietic action when given in small quantities as a supplement to iron. Waddell, Steenbock, and Hart (1) have tested all of the metals that have been reported to have such a positive action and, even though they fed the metals at lower and at higher levels than those used by other workers, their conclusion was that copper alone has a specific action in the cure of nutritional anemia. As the results obtained by these different workers leaves doubt as to whether or not metals other than copper have any action in stimulating hemoglobin regeneration, we felt that there would be greater certainty of demonstrating a slight curative action which any of these metals might have, if we used them in relatively large quantities. Based upon the feeding of cobalt, nickel, zinc, and manganese at the higher levels used by Waddell, Steenbock, and Hart (1), our experimental results completely support their conclusion concerning the specificity of copper as a supplement to iron in the cure of nutritional anemia.

Although there is no reason to believe that an animal from one compartment of the cage was able to obtain metals from the supplements used in the adjacent compartments, it is interesting to note that throughout the experiment until death Rat 14 (Group VI, Chart II) on zinc occupied a cage compartment between Rats 13 and 15 (Group VIII, Chart III) on supplements of copper. The fact that this rat failed to show more than the slight temporary regeneration of hemoglobin is evidence that no "cross-contamination" between compartments occurred.

It may be well to state that no attempt was made in our study to ascertain the lowest level of copper that would be effective in the cure of nutritional anemia.

SUMMARY

1. A glass cage for use in the study of metals in nutritional anemia of the rat is described.

2. By the technique employed, a nutritional anemia was successfully produced in first generation rats.

3. Purified iron alone is ineffective in preventing or curing the nutritional anemia produced in rats by a diet of whole milk.

4. Cobalt, nickel, zinc, and manganese as supplements to iron fail to cure such a nutritional anemia.

5. Copper alone, of all the metals studied, has the ability to supplement iron in curing the nutritional anemia of rats.

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THE INFLUENCE OF INSULIN ON GLYCOGEN DISTRIBU-
TION IN MARINE FISHES

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INTRODUCTION

The problem of the mechanism of insulin action has received considerable attention in the past few years. Explanations have been sought in several ways, particularly through the study of the effect of insulin on blood sugar, glycogen distribution, the respiratory quotient, and, more recently, on the intermediary metabolism of the carbohydrates. A few investigators have also been concerned with the influence of insulin on protein, fat, and mineral metabolism. An excellent review of literature up to 1926 has been given by Macleod (1). The more recent work has been summarized by the same author in a series of lectures delivered before the London Hospital (2).

Cori and Cori, Macleod and his coworkers, and Lesser and collaborators have made many significant contributions to the specific problem of the influence of insulin on glycogen distribution. Their work has been restricted almost entirely to mammals. We find this same thing to be true of the contributions of other workers. The data obtained have been more or less conflicting, and we believe that it can be safely stated that we have not yet arrived at a point where final conclusions can be drawn. The whole insulin problem is complicated by the fact that the physiological response of animals to insulin seems to differ according to whether they are diabetic, starved, or are absorbing carbohydrates. There seems, also, to be an additional factor, that of the general metabolism of the experimental animals. These, and perhaps other complications, are no doubt responsible in a large measure for the several conflicting theories of the action of insulin. Goldblatt classifies the prevalent theories in a recent paper (3).

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In view of the more or less conflicting results that have been obtained in mammals, it was thought advisable to attempt a study on some cold-blooded animal, in which the general metabolic activity is considerably slower. Accordingly fishes were chosen and a series of experiments on them conducted.

We feel that the lower metabolism of fishes makes them more favorable for this study than mammals in that changes due to the action of insulin take place more slowly and the details can be more easily detected.

The work was carried out during the summer of 1929 at the United States Fisheries Laboratory at Woods Hole, Massachusetts. We are especially grateful to Dr. O. E. Sette for placing the facilities of the laboratory at our disposal.

Procedure

In a previous paper, Gray and Hall (4) have shown that insulin shock may be easily produced in species of active fishes. However in sluggish forms there is little external evidence of the action of insulin as convulsions. It was also shown that in fishes a much larger dosage is necessary to produce convulsions and lowering of blood sugar concentration than is required for mammals.

Since the scup, *Stenotomus chrysops*, L., was used in these previous studies, and much information concerning its reaction to insulin had already been obtained, this fish was chosen as the experimental animal. The fishes were either taken from commercial traps or caught by hook and line and brought to the laboratory, where they were kept in hatching boxes for about 24 hours before using. These boxes were supplied with an abundance of running sea water to prevent any asphyxial conditions. The practice of keeping the fishes in these boxes for some time before use is necessary to insure more constant physiological conditions and less individual variation (5). The fishes were divided into two groups and an attempt was made to have animals of about the same weight in each group. The average weight of the animals was about 250 gm. One group was used as a control. At a set time 10 units of insulin (Lilly) were injected intraperitoneally into each of the animals in the experimental group. The control animals were handled in two ways: they were either injected with a volume of physiological saline equal to the volume of insulin, or they were

left alone until killed for blood sugar and glycogen determinations. The reasons for the injection of saline will be brought out later. After recorded intervals, following the injection with insulin, one. fish from the experimental and one from the control group were used for blood sugar and glycogen determinations. The fishes were quickly removed from the hatching boxes and blood from the severed caudal artery collected in oxalated tubes. The spinal cord was then severed just back of the brain to prevent muscular movements, and the body cavity opened. The liver was removed *in toto* by grasping it with forceps at its point of attachment and immediately dropped into a tared flask containing the proper amount of hot 60 per cent KOH. After this a strip of muscle was taken from one side of the animal and dropped into another tared flask containing hot KOH. The entire operation consumed less than 2 minutes. The weight of the liver and muscle was determined by reweighing the tared flasks on precision balances. The weight of the entire fish was determined by weighing the remains and adding to the result the weight of liver, muscle, and blood removed. The blood sugar was determined according to the Folin modification of the Folin-Wu method (6); the glycogen according to the well known method of Pflüger (7). Instead of the glycogen being read directly with a polariscope it was inverted to glucose with 2.2 per cent HCl and the glucose determined by the Hagedorn-Jensen method (8). The results were expressed in terms of glycogen by using the formula, glycogen = glucose × 0.927.

Animals were bled and analyses were made on the 1st, 2nd, 3rd, 4th, 5th, 6th, 8th, 10th, 12th, and 14th hours after the injection of insulin. The data obtained from the analyses of several repeated series were then graphed to show the relation of the blood sugar, muscle, and liver glycogen in the insulinized animals to the same constituents in uninsulinized controls.

Results

Fig. 1 summarizes the results obtained in this study. The data from the insulinized fishes are expressed in percentage variation from normal concentrations found in the controls. These figures are plotted against time after the fishes were given insulin. Each curve is a composite of results obtained from 32 individuals. The average normal concentration of the liver glycogen was found to

be 33.07 mg. per gm. of liver, of muscle glycogen 0.883 mg. per gm. of muscle, of blood sugar 46 mg. per 100 cc. of blood.   In the insulinized fishes the blood sugar concentration is above normal
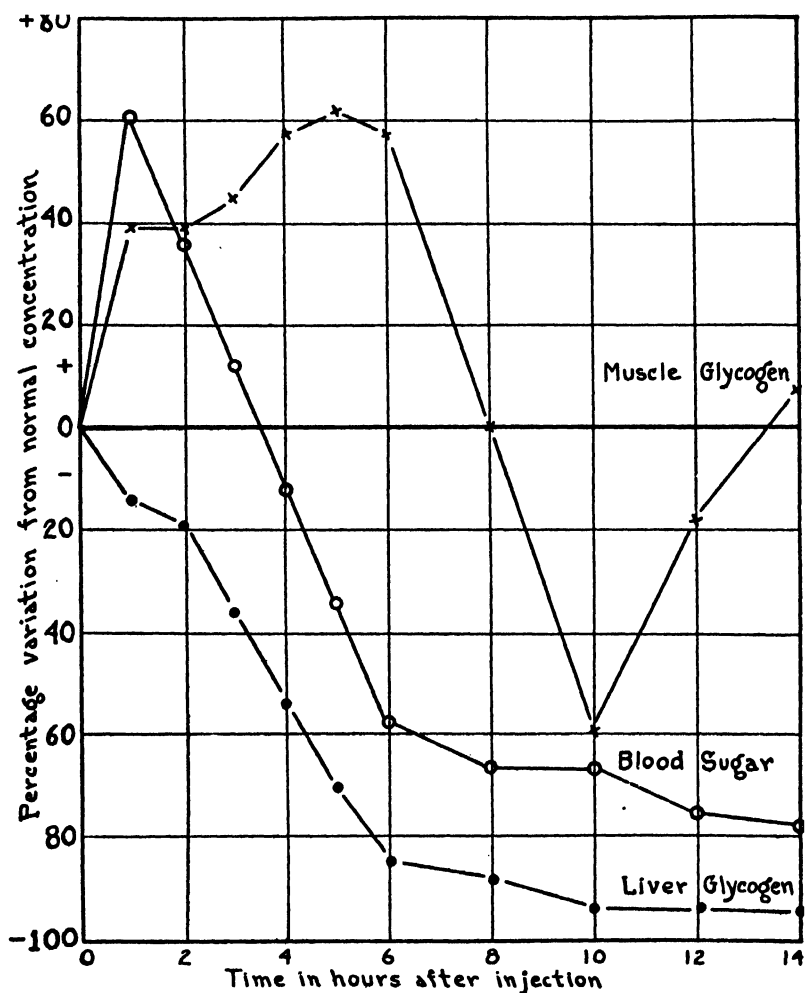
FIG. 1. The influence of insulin on carbohydrate distribution in the scup

for a period of 3 hours after the insulin injection.   This period of hyperglycemia is followed by prolonged hypoglycemia, the blood sugar falling to a very low level.   The liver glycogen decreases in

concentration from the start, and continues to decrease until only about 5 per cent of the normal amount remains in the liver. The muscle glycogen, on the other hand, rises gradually upon injection of insulin, and reaches the peak of its concentration between the 4th and 5th hours after the injection; there then follows a very decisive drop in concentration. The sudden drop occurs at a time when convulsive symptoms are noted in the animals. The lowest level of concentration is found at the 10th hour after insulin administration; from that point on to the 14th hour the concentration increases again until it is approximately normal.

DISCUSSION

The results indicate that insulin effects a marked change in the distribution of glycogen in the scup. Concomitant with the drop in liver glycogen and blood sugar there is a rise in muscle glycogen, indicating that insulin may bring about an excessive storage of muscle glycogen at the expense of liver glycogen and blood sugar. It is impossible to say that all the carbohydrate thrown out of the liver and blood is accounted for by storage in the muscles. A considerable part of it may have been oxidized, or have been taken care of in some other manner.

The hyperglycemia observed following immediately after the insulin injections is of interest. It was thought that this might be a result of the act of injecting the fishes. As a control some of the fishes were injected with a volume of physiological saline equal to the volume of insulin given the experimental animals. The results were not entirely negative. A slight hyperglycemia followed in the control animals, but it was never as marked as in the experimental animals. It appeared that the insulin did have a definite hyperglycemic effect. We suggest the following interpretation of this situation. The injected insulin stimulates liver glycogenolysis. Immediately following administration of insulin, on account of the high initial concentration of liver glycogen, a large quantity of sugar is supplied to the blood. For a time more sugar is acquired by the blood than is removed by the tissues through oxidative and other processes. However, as the glycogen concentration of the liver gradually decreases, less and less sugar is supplied by the liver, until finally a stage is reached where the supply no longer exceeds the demand and hypoglycemia sets in.

Collens and Murlin (9) working with dogs found that portal injection of insulin caused a marked hyperglycemia within 5 minutes after the injection. The hyperglycemia lasted for only a short time and was followed by the usual hypoglycemia. They attributed the temporary hyperglycemia to rapid initial glycogenolysis in the liver.

The concentration of muscle glycogen appears to be a sensitive indicator of the convulsive stage in insulinized fishes. It will be noted from Fig. 1 that muscle glycogen concentration gradually increased for about 6 hours after insulin injection and then suddenly dropped. This drop occurred at a time when convulsive symptoms were noted in the fishes. At that time they would swim with great rapidity, going in extremely haphazard fashion, and striking the sides of the tank. This period of rapid propulsion was followed by a quiescent period, with the fishes resting bellies upward. At the 10th hour the fishes were in the most pronounced stage of convulsions, and the lowest muscle glycogen concentrations were then recorded. At the 12th and 14th hours the fishes showed only slight convulsions and higher muscle glycogen concentrations. At the same time however, blood sugar and liver glycogen do not increase in concentration, in fact the blood sugar concentration drops even lower than it was at the 10th hour. Thus it is difficult to say whether fishes at the 12th and 14th hours were past the critical stage of insulin convulsion, or were merely less susceptible to the insulin than fishes at the 10th hour. One might think that there would be a rise in blood sugar if the fishes were recovering from the effects of the insulin. If our interpretation rested only on external evidence and the determination of the muscle glycogen concentration, we might say that the fishes were recovering from insulin shock; but the blood sugar concentration does not indicate such a situation. On the other hand, perhaps, in cases of recovery from insulin shock, muscle glycogen recovers before liver glycogen and blood sugar. If this were true our interpretation would seem consistent.   .

Cori and Cori (10), who have worked extensively on rats, came to the conclusion that there is a cycle of the glucose molecule in the body. They think that insulin is of significance in that it accelerates the cycle in the direction of blood glucose to muscle glycogen. Acceleration in this direction leads to hypoglycemia,

and, secondarily, to a depletion of the glycogen stores of the liver. Barbour *et al.* (11), working with the standard white rat, concluded that large doses of insulin injected into fed rats have an inhibitory effect on glycogen formation in the liver, at the same time increasing glycogen in the muscles, the total gained by the muscles being of about the same magnitude as that lost in the liver. Smaller doses, while having the same effect on the liver glycogen, produce no demonstrable change in muscle glycogen. In fasted rats they found that insulin always caused a decrease in both liver and muscle glycogen, but before there was any demonstrable recovery in blood sugar the liver glycogen concentration returned to, or about, the general level. Choi (12) found that muscle glycogen increased when glucose and insulin were injected together. Markowitz *et al.* (13), working with the dog, found that a rise in muscle glycogen could not be demonstrated in dogs with excised liver and pancreas. If insulin was injected, or if the liver alone was excised the muscle glycogen increased. We feel that Markowitz and his coworkers have performed a critical experiment, in that they demonstrate, in a seemingly irrefragible manner, the ability of insulin to bring about the storage of glycogen in the muscles.

We cite the previous experiments for the purpose of illustrating the general agreement of the results we have obtained with fishes with those obtained with mammals. Our results also agree with von Issekutz and Végh (14), and with Takuwa (15), who worked with turtles. They found a decrease in liver glycogen after the injection of insulin.

Conclusions derived from studies on the action of insulin have been very conflicting. Some authors have obtained results that appear to be quite contradictory to those obtained by others. Obviously, our results cannot be in agreement with all.

Rather than review all of the contradictory interpretations of insulin action the reader is referred to Macleod (1, 2). Especial mention, however, should be made of a paper by Goldblatt (16). This author worked with young rabbits and obtained results that led him to conclude that the action of insulin is to "lock" glycogen in the liver. We did not find evidence of such a phenomenon in the scup. Consequently, we are somewhat skeptical of Goldblatt's interpretation, not only on account of our inability to confirm his conclusion, but also because of evidence presented by other investigators.

In conclusion we would like to suggest a possible explanation to account for the conflicting evidence as to the action of insulin which seems to prevail at the present time. Practically all of the work concerned with the mechanism of insulin action has been carried on with homeothermic animals and scarcely any with poikilothermic animals. The metabolism of a homeothermic animal is much more rapid than that of a poikilothermic animal. Changes go on so rapidly in mammals that some of them may be missed entirely. As proof of the more rapid action of insulin in the homeotherms, we point out that 1½ units of insulin per kilo of body weight injected into a rabbit will bring on convulsions in about 5 hours, while the fishes used by us were receiving about 40 units of insulin per kilo of body weight and yet convulsions did not occur until 8 to 10 hours after they were injected. We feel that, due to the slower metabolism, a poikilotherm is more favorable to employ in the study of insulin action. The results are brought to us in the form of a slow motion picture, and the details of the action can be studied with greater facility.

SUMMARY

1. Massive injections of insulin (about 40 units per kilo of body weight) elicit a marked change in glycogen distribution in normal fasting scup.

2. Following the injection glycogen is thrown out of the liver, and after a transient hyperglycemia, pronounced hypoglycemia occurs. Concomitant with the drop in liver glycogen concentration and blood sugar concentration, there is a rise in muscle glycogen until convulsive symptoms appear in the animals. The concentration of muscle glycogen then falls rapidly.

3. It appears that insulin causes an increase in storage of muscle glycogen at the expense of liver glycogen and blood sugar.

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SYNTHETIC RATIONS AND HEMOGLOBIN BUILDING

A NOTE ON THE DRABKIN-WAGGONER MODIFICATION OF THE BIAZZO METHOD FOR DETERMINING COPPER*

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In 1929 Drabkin and Waggoner (1) published a short paper in which they demonstrated that a nutritional anemia induced in rats by a whole milk diet could be cured by transferring the rats to a synthetic diet containing iron but which they claimed to be copper-free. If it can be demonstrated without doubt that their synthetic ration is absolutely copper-free, then other factors must play the same rôle in hemoglobin synthesis that copper does. Attempts in this laboratory (2) to demonstrate the activity of elements other than copper in hemoglobin regeneration have been essentially negative.

We immediately questioned the complete absence of copper in a synthetic ration compounded from the materials used by Drabkin and Waggoner because in our work with chicks we had found it very difficult to prepare synthetic rations exceedingly low in copper (3). At that time we analyzed a sample of their ration, which was kindly furnished us by Dr. Drabkin. The sample sent to us was designated as "copper-free Ration 1," but in a later publication these workers refer to it as "low copper Diet 1." The copper was determined by the Biazzo method as modified by Elvehjem and Lindow (4) and 10 gm. of the ration were found to contain 0.023 mg. of Cu. If their rats consumed 10 gm. of this synthetic ration per animal per day, then each rat ingested daily sufficient amounts of copper for normal hemoglobin formation.

In a recent publication (5) Drabkin and Waggoner criticize

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the accuracy of our determination and claim to have obtained hemoglobin regeneration in anemic rats by the use of a more highly purified synthetic ration, "low copper Diet 2." This ration, according to their analysis, contained exceedingly low amounts of copper, namely 0.0019 mg. of Cu per 10 gm. of ration, a figure which was obtained from "an average of eight analyses, three of which were 0.000 mg. of copper, while two were 0.004 and 0.005 mg. respectively." If figures which vary to such a large degree as these can be accepted, and if their method of determining copper is analytically sound, then the copper content of the daily ration would be lower than that of 40 cc. of cow's milk, the approximate daily consumption of a young rat restricted to such a diet. Our recent analyses show that cow's milk contains from 0.15 to 0.20 mg. of Cu per liter (6).

Although their methods of animal experimentation are open to criticism, because the rats were started on the synthetic diets before they were distinctly anemic and because no record of animals receiving only purified iron plus milk is reported as a control, which is necessary to demonstrate that copper from other sources is not affecting the results, we will not consider these facts in this short note, but will only discuss the analytical methods used by these workers.

We wish to demonstrate that the method of Elvehjem and Lindow for the determination of copper in biological materials gives accurate results and that the modified method suggested by Drabkin and Waggoner will give correct results only when the proper precautions are taken. Drabkin and Waggoner state that ferric iron is an interfering substance in the development of the color of the copper-thiocyanate-pyridine complex, and present a detailed table giving determinations which show how the addition of iron increases the copper reading. These results (Table III of their paper (5)) are obtained exclusively by the use of acidified water solutions of iron and copper. We do not question the possibility of securing the published results under such conditions, but in a determination of copper in the ash of biological materials· which always contain phosphates, there will be no interference due to iron unless it is present in excess of the phosphate. This fact was recognized by Elvehjem and Lindow (4) in the development of the Biazzo method and led them to state that, "Ferric iron,

however, when present in large amounts will give a brownish tinge to the chloroform layer, but out of a hundred food materials thus far analyzed this difficulty has been encountered in only a few cases. Animal tissues which are especially high in hemoglobin and commercial products made from them are probably the only samples which will give this trouble."

Drabkin and Waggoner added sodium pyrophosphate to prevent the interfering action of iron when they analyzed their rations because, according to Warburg (7), pyrophosphate forms with iron an unionized compound and thereby prevents its activity while copper does not form such a compound. Although pyrophosphate itself does not affect the activity of copper, it may have an interfering action when fairly large amounts of iron are present. In a detailed study of the activity of copper, in pyrophosphate solutions containing varying amounts of iron, in the catalytic oxidation of cysteine made by one of us (8), it was found that the presence of iron inhibited the activity of the copper to a large degree and that the amount of inhibition was dependent upon the pH and temperature of the solution. When an acid solution of ferric iron and sodium pyrophosphate is neutralized with sodium hydroxide a precipitate is formed which dissolves in acetic acid with difficulty. This precipitate often occludes a portion of the copper present, thus preventing its quantitative estimation. Also if a neutral solution containing copper sulfate and an excess of sodium pyrophosphate is acidified with acetic acid a precipitate of copper pyrophosphate is formed when a pH of 4.0 is reached. Therefore it is not surprising to find that the results of Drabkin and Waggoner vary from 0.000 to 0.005 mg. of Cu per 10 gm. of different samples of the same ration when pyrophosphate is used.

We had previously found (unpublished data) that in the determination of copper in blood with the use of pyrophosphate much better results were obtained when NaOH was replaced by NH$_4$OH, since no precipitate of ferric pyrophosphate was formed in the presence of ammonia. When analysis of Ration 1 (low copper Diet 1) was repeated, this procedure being used, results varying from 0.010 to 0.017 mg. of Cu per 10 gm. of ration were obtained. The results were variable and somewhat lower than 0.023 to 0.025 mg. which we obtained without pyrophosphate. However when certain precautions as to pH and temperature were taken

figures identical with the unmodified Elvehjem-Lindow method, namely, 0.023 to 0.025 mg. of Cu per 10 gm. of ration, were obtained. The optimum pH and temperature condition can be assured by the following procedure. The HCl solution of the ash is evaporated to a volume of 15 cc., 1 cc. of 4 per cent $Na_4P_2O_7$ is added, the solution is neutralized to phenolphthalein with concentrated NH_4OH, and made acidic with acetic acid. When the rather concentrated acid solution is neutralized with concentrated NH_4OH, the temperature is increased sufficiently (approximately 40°) to keep all the copper in solution. The addition of acetic acid should be regulated so that the pH of the solution does not

TABLE I

Effect of Various Modifications of the Elvehjem-Lindow Method on Recovery of Copper in Solutions Containing Relatively Large Amounts of Ferric Iron

Each analysis was made on an HCl solution containing 0.03 mg. of Cu, 0.5 mg. of Fe as $FeCl_3$, and 50 mg. of Na_2HPO_4.

Neutralized with NaOH	Neutralized with NH_4OH	4 per cent $Na_4P_2O_7$ added	Temperature and pH controlled	Cu found
		cc.		*mg.*
+		None	−	0.031
+		"	−	0.030
+		1	−	0.016
	+	None	−	0.030
	+	1	−	0.024
	+	1	+	0.029
	+	1	+	0.030

fall below 4.0 when the color is developed. Only 30 per cent of the copper can be estimated if the pH falls to 3.4.

In Table I are given the figures for the recovery of copper in solutions containing copper, iron, and orthophosphate when different procedures are used. The figures clearly show that copper can be determined accurately in the presence of rather large amounts of ferric iron when orthophosphates are present. As soon as 1 cc. of pyrophosphate is added low results are obtained whether the acid solution is neutralized with NaOH or NH_4OH. However, when the temperature and the pH are controlled the copper is recovered quantitatively.

The figures obtained for 10 gm. of Drabkin and Waggoner's

Ration 1 (low copper Diet 1) when similar procedures are used are given in Table II. When 1 cc. of sodium pyrophosphate is used the copper found is reduced from 0.022 to 0.025 mg. to 0.015 mg. This figure is a typical result, but often lower figures of the same magnitude as those given by Drabkin and Waggoner were obtained. When the temperature and pH were controlled, figures of 0.022 to 0.025 were again obtained in the presence of pyrophosphate. Complete recoveries of added copper were also obtained when this method was used.

These analytical findings make it evident that the copper content of Drabkin and Waggoner's synthetic Ration 1 (low copper

TABLE II

Effect of Various Modifications of the Elvehjem-Lindow Method on Recovery of Copper in Acid Solutions of Ash from Drabkin and Waggoner's Low Copper Diet 1

Each analysis was made on the ash of 10 gm. of sample.

Neutralized with NH₄OH	4 per cent Na₄P₂O₇ added	Temperature and pH controlled	Cu added	Cu found
	cc.		mg.	mg.
+	None	−		0.022
+	1	−		0.015
+	1	+		0.025
+	1	+		0.024
+	1	+	0.02	0.042
+	1	+	0.02	0.043
Figures for same ration obtained by Drabkin and Waggoner..				0.0043-0.0075

Diet 1) is approximately 3 times as high as reported by them; namely 0.022 to 0.025 mg. instead of 0.0043 to 0.0075 mg. of copper per 10 gm. These figures place the daily copper intake of a rat eating 10 gm. of such a ration at 3 to 4 times the amount ingested when it was restricted to 40 cc. of whole cow's milk, which would furnish approximately 0.006 to 0.008 mg. of copper.

Drabkin and Waggoner (5) also report that a ration (low copper Diet 3) has been prepared which is exceedingly low in copper and which is still very active in promoting hemoglobin regeneration. They claim that this ration is so low in copper that 10 gm. of the ration does not yield sufficient copper for analysis. We have also

analyzed this ration (a sample of which was sent to us by Dr. Drabkin) using the pyrophosphate method of Drabkin and Waggoner under properly controlled conditions, and the copper content was found to be 0.017 mg. per 10 gm. of ration. This ration is only slightly lower in copper than low copper Diet 1 and therefore also furnishes sufficient copper for normal hemoglobin formation.

Our copper data on the Drabkin-Waggoner rations clearly explain why they had hemoglobin restoration in anemic rats transferred to their synthetic "low" copper diets. Further, we believe that our analytical data restore in principle our findings on the relation of copper to iron for hemoglobin building.

SUMMARY

Figures are presented to show that the use of pyrophosphate for preventing the interfering action of large amounts of ferric iron in the determination of copper by the Elvehjem and Lindow method may lead to a serious loss of copper. The difficulty encountered by the use of pyrophosphate may be eliminated if the temperature and the pH are controlled.

Analyses of Drabkin and Waggoner's low copper diets demonstrate that they do contain sufficient amounts of copper to account for the activity of these rations in hemoglobin regeneration.

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THE EFFECT OF LARGE DOSES OF IRRADIATED ER-
GOSTEROL UPON THE ASH CONTENT OF THE
FEMORA OF YOUNG AND ADULT RATS

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Recent work has demonstrated that enormous doses of irradiated
ergosterol may produce a hypercalcemia and abnormal calcifica-
tion of body tissues. The source of this apparent excess of cal-
cium has not been definitely determined. Several papers have
recently appeared in which data have been presented indicating
that activated ergosterol, if administered in very large amounts,
withdraws calcium from bone. Hess, Lewis, and Rivkin (1),
Light, Miller, and Frey (2), Brown and Shohl (3), and Shohl, Gold-
blatt, and Brown (4) have shown that the percentage of ash in
the bones of growing rats is less in those animals receiving toxic
amounts of irradiated ergosterol than in the bones of animals re-
ceiving either a stock ration or a diet supplemented with non-toxic
doses of vitamin D preparations. In each case the conclusion
has been made that the difference in the amount of bone ash is
due to withdrawal of calcium salts from the bone. These authors
do not consider the possibility of a difference in the increase in the
percentage of ash in the growing bones during the experimental
period. The normal ash content of the fat-free femora of rats of
4 weeks of age is approximately 40 per cent. On a stock diet, and
especially if this diet is supplemented with liberal but not toxic
amounts of irradiated ergosterol, the percentage of ash in the long
bones of rats rapidly increases during the first few weeks after
weaning. If, however, the amount of ergosterol given is sufficient
to produce toxic symptoms with a loss of appetite and a cessation
of growth, it is possible that there is an interference with the nor-
mal deposition of the calcium salts in the bone. This would be

especially true in those cases where the food consumption is considerably reduced with a consequent lack of calcium and phosphorous. The animals receiving the smaller amounts of vitamin D would, on the other hand, be growing normally and in the course of 2 or 3 weeks the bones of these animals would contain considerably more ash than the bones of the animals poisoned by large doses of vitamin D.

If large amounts of irradiated ergosterol can actually withdraw calcium from bone it should be possible to demonstrate a decrease in the ash content of bones of adult rats. In the following experiments such an attempt has been made.

EXPERIMENTAL

Adult rats whose previous history as to parentage and age was known were used. The following food mixture, which is a modification of the Steenbock (5) stock ration, was employed as the basal diet.

	per cent
Yellow corn	71.0
Linseed oil meal	16 0
Crude casein	5.0
Ground alfalfa	2.0
Whole milk powder	5.0
Sodium chloride	0.5
Calcium carbonate	0.5

The animals were placed in separate cages at the beginning of the experiment. Each experimental animal had an individual control. The control was always of the same litter and usually of the same sex as the experimental animal. The latter precaution was taken because Hammett (6) has shown that up to at least 150 days of age the femora and humeri of female rats have relatively more ash than males of the same age. The amount of food consumed by each experimental animal was determined daily, and an equal amount of food was given to its control for the following 24 hours. The irradiated ergosterol used was of two different commercial preparations. The preparations are referred to in Table I as Samples 1 and 2. Sample 1 according to the manufacturers had an antirachitic potency of 1500 D[1] (1500 × the vitamin

[1] Kindly furnished by the Winthrop Chemical Company, Inc., New York.

TABLE I

Influence of Large Doses of Irradiated Ergosterol upon Ash Content of Femora of Adult Rats

Rat No.	Age	Sex	Ergosterol sample No.	Daily dose of ergosterol*	Days on experiment	Remarks	Per cent ash		Average
							Right femur	Left femur	
	days			*cc.*					*per cent*
1	175	F.	1	600	10	Died		58.74	58.74
2	175	"		0	10	Killed	59.32	59.32	59.32
3	186	M.	1	600	7	Died	63.62	63.89	63.75
4	186	"		0	7	Killed	63.96	63.94	63.95
5	155	F.	1	600	8	Died	63.02	62.92	62.97
6	155	"		0	8	Killed	64.12	63.38	63.75
7	147	M.	1	600	7	Died	64.12	63.83	63.97
8	147	"		0	7	Killed	63.74	64.01	63.87
9	141	F.	1	600	11	Died	62.05	62.16	62.10
10	141	"		0	11	Killed	65.49	66.17	65.83
11	141	"	1	600	10	Died	63.11	62.76	62.93
12	141	"		0	10	Killed	64.94	64.34	64.64
13	152	M.	2	400	23	"	66.49	66.91	66.70
14	152	F.		0	23	"	64.98	65.94	65.46
15	152	M.	2	400	23	Died	60.47	60.74	60.60
16	152	"		0	23	Killed	65.03	65.24	65.14
17	100	"	2	500	12	"	61.60	62.18	61.89
18	100	"		0	12	"	61.73	61.89	61.81
19	100	F.	2	500	7	Died	63.99	63.15	63.57
20	100	"		0	7	Killed	64.94	64.68	64.81
21	150	"	2	500	12	"	60.82	60.92	60.87
22	150	"		0	12	"	59.53	60.99	60.26
23	150	M.	2	500	11	Died	61.74	61.74	61.74
24	150	F.		0	11	Killed	61.54	59.41	60.47
25	135	M.	2	500	12	Died	62.31	63.09	62.70
26	135	"		0	12	Killed	63.84	63.36	63.60

TABLE I—*Concluded*

Rat No.	Age	Sex	Ergos-terol sample No.	Daily dose of ergos-terol*	Days on experi-ment	Remarks	Per cent ash		Average
							Right femur	Left femur	
	days			*cc.*					*per cent*
27	135	M.	2	500	10	Died	63.97	63.82	63.89
28	135	"		0	10	Killed	64.29	64.20	64.24

Average, rats given irradiated ergosterol	62.60
" control rats	63.36

29	175	M.	1	600	10	Died	56.78	57.94	57.36
30	186	F.	1	600	9	"	64.67	63.94	64.30
31	155	"	1	600	6	"	63.91	63.99	63.95
32	152	"	2	400	23	Killed	60.27	60.61	60.44
33	100	"	2	500	4	Died	62.09	61.46	61.77

Average	61.65

Animals 29 to 33 received ergosterol; no paired controls.

* Dose of irradiated ergosterol is expressed in cc. of the antirachitic equivalent of Steenbock standard cod liver oil.

D content of the Steenbock standard cod liver oil) and Sample 2, 10,000 D^2. The ergosterol was given by mouth to the animals from a Luer tuberculin syringe. The preparations were so diluted with cottonseed oil that each animal received from 0.1 to 0.4 cc. of solution. Each control received an amount of cottonseed oil equal to the total quantity of oil given the corresponding experimental animal. With the exception of Rats 13 and 15 (Table I) the administration of irradiated ergosterol was continued for a period of not more than 11 or 12 days. When, as frequently happened, an experimental animal died before this, its control was immediately sacrificed. Otherwise both were killed at the end of this period. Rats 13 and 15 were given irradiated ergosterol for 23 days.

All the animals receiving the preparations of irradiated ergosterol showed the characteristic symptoms of vitamin D hypervitaminosis; *viz.*, loss of appetite, emaciation, and the development of a greasy rough coat with the fur turning yellow about the genitals.

As soon as possible after the death of an animal (immediately following death in those cases in which the animal was killed) both femora were removed, thoroughly cleaned, and dried. At the same time certain tissues were removed for histological examination in order to show that the animals had received sufficient irradiated ergosterol to produce the usual pathological changes. The details of this procedure with the results are given at the end of this article.

After drying, the bones were extracted with 95 per cent ethyl alcohol in a Soxhlet extractor, thoroughly dried and weighed, and the percentage of total ash determined. The results of these analyses are given in Table I. The animals are arranged in pairs. The first member of each pair is an experimental animal (one receiving irradiated ergosterol) and the other is its control. The ages of the animals are given at the beginning of ergosterol administration. The daily dosage of ergosterol is expressed in cc. of the antirachitic equivalent of standard cod liver oil. Since 75 mg. of standard cod liver oil constitute 1 rat unit (Steenbock) the approximate number of rat units each experimental animal received daily may be obtained by multiplying the numbers in the dose column by 13. Under the column headed "Remarks" it is stated whether the animal died or was killed. In the majority of cases the experimental animal died of overdosage of ergosterol. Those few animals which did not die, but instead were killed with chloroform, showed to an extreme degree the characteristic symptoms of vitamin D hypervitaminosis.

As seen from Table I there is no constant difference in the percentage of ash of the femora of the animals receiving ergosterol and of their paired controls. Among the experimental animals the ash varies from 58.74 to 66.70 per cent with a mean of 62.60 per cent. The controls show a variation from 59.32 to 65.46 per cent with a mean of 63.36 per cent. This is a difference of only 0.7 per cent in the average of the two groups, which is vastly different from that reported by other investigators, the maximum of about 15 per cent being given by Brown and Shohl. In one or two cases only is there an appreciable difference between the femora of an experimental animal and those of its control. There is a difference of about 4½ per cent between the ashes of the femora of Rat 15 and its control Rat 16. However, when Rat 15 is com-

pared with Rat 13 it is noted that there is a difference of over 6 per cent in the ashes of the femora.   These two animals had identical treatment throughout their entire life span.   They were litter mates of the same sex with the same preexperimental and experimental treatments.   They died on the same day.   In spite of the similarity in sex, age, parentage, and treatment of these two animals there was a marked difference in the amount of ash in the femora.   This difference is not due to errors in the determination as is shown by the close agreement of the percentage of ash of the right and left femora of each animal.   The wide variation in the ashes of the femora of these two animals not only shows that it is impossible to draw conclusions from a few animals, but that the difference between the ash contents of the femora of Rats 15 and 16 is probably due to individual variations and not to the influence of irradiated ergosterol.

In the latter part of Table I are given the results of similar experiments on five animals which did not have paired controls. Here again there is no marked decrease in the percentage of bone ash comparable with that reported in the papers referred to above. It is apparent from these experiments that doses of irradiated ergosterol sufficiently large to produce the usual symptoms of vitamin D hypervitaminosis, and in many cases death, do not materially reduce the amount of bone ash in the femora of adult rats.

.   If large doses of irradiated ergosterol can withdraw calcium from the bones of young rats, the amount of ash in the bones after feeding the ergosterol should be less than before the ergosterol was administered.   Light and associates (2) and Brown and Shohl (3) do not give any analyses made at the beginning of their experiments.   Instead they draw their conclusions from observed differences in the ash content of bones taken from young rats that had been fed varying amounts of irradiated ergosterol.   They entirely neglect the fact that under the influence of large but non-toxic doses of vitamin D, calcification of the young growing bone is rapid.   Brown and Shohl increase this discrepancy by keeping their animals receiving the non-toxic doses on experiment for 3 weeks; whereas they kill the animals receiving toxic amounts in 11 or 14 days.   Hess and associates (1) do not state at what period in their experiments the bones of the control animals were taken for analysis.

TABLE II

Influence of Large Doses of Irradiated Ergosterol upon Ash Content of Femora of Growing Rats

Rat No.*	Sex	Daily dose of ergosterol†	Days on experiment	Remarks	Per cent ash		Average
					Right femur	Left femur	
		cc.					*per cent*
34	M.	250	4	Died	40.37	40.14	40.26
35	F.	250	9	Killed	42.34	41.03	41.69
36	M.	250	4	Died	40.64	40.46	40.55
37	"	0	0	Killed	40.65	40.57	40.61
38	"	0	0	"	42.29	42.50	42.39
39	"	0	0	"	42.99	42.19	42.59
40	"	200	10	Died	38.74	38.08	38.41
41	F.	200	5	"	45.09	42.95	44.02
42	"	0	0	Killed	42.24	42.37	42.30
43	"	0	0	"	42.61	44.15	43.36
44	M.	20	15	"	51.44	52.88	52.16
45	F.	20	15	"	51.48	51.67	51.58
46	M.	200	14	"	38.43	38.20	38.32
47	F.	200	10	Died	38.96	39.30	39.13
48	"	0	0	Killed	42.72	42.57	42.64
49	"	0	0	"	44.87	42.79	43.83
50	M.	20	15	"	52.98	52.22	52.60
51	F.	20	15	"	52.84	53.39	53.11
52	M.	200	6	Died			42.09‡
53	"	200	8	"			43.76‡
54	F.	200	8	"			43.28‡
55	M.	0	0	Killed			43.68‡
56	F.	0	0	"			40.69‡
57	M.	0	0	"			41.94‡

Average, rats given irradiated ergosterol.................... 41.15
    "   control rats....................................... 42.38

* These animals are grouped according to litters. Their ages varied from 28 to 30 days at the beginning of the experiment.

† Dose of irradiated ergosterol is expressed in cc. of the antirachitic equivalent of Steenbock standard cod liver oil. Sample 2 was fed to these animals.

‡ Right femur only.

In one of the experiments of Shohl, Goldblatt, and Brown (4)
twelve rats of the same litter, 7 weeks of age, were divided into
six groups of two animals each.   One group was killed as controls.
Four of the groups were given irradiated ergosterol for varying
lengths of time ranging from 1 to 7 days, and the remaining group
was given unirradiated ergosterol.   The femora of the first group
had an ash content of 51.3 per cent and those of the last group 56.6
per cent.   The figures for the ash content of the femora of all the
rats which received the vitamin D preparation fell between the
above values.   These results would seem to indicate that large
doses of irradiated ergosterol can prevent the normal calcification
of bone, but do not produce a demineralization by actually with-
drawing calcium salts from the bone.

Several experiments were conducted to determine if large doses
of irradiated ergosterol can reduce the amount of ash in the bones
of growing rats.   The results are summarized in Table II.   The
first experiment was conducted on six rats of the same litter.
Three of the rats were killed when 28 days of age while the other
three rats were given irradiated ergosterol in large amounts.   The
rats receiving the vitamin D preparation showed anorexia after
the 1st day of vitamin D administration.   As seen from Table II
there is no marked difference between the ash content of the
femora of the control animals and of the animals which received
the irradiated ergosterol.

This experiment was repeated with two litters of six animals
each.   Four of the twelve animals (Table II, Rats 40 to 51)
were killed when 30 days of age, four were given toxic doses of
vitamin D, and the remaining four rats were given rather large but
non-toxic doses of irradiated ergosterol.   In the case of these last
four animals the ergosterol preparation was mixed into the diet
and the doses in Table II are given on the assumption that each
animal ate an average of 10 gm. of the ration daily.

In contrast to the experiment conducted with Rats 34 to 39,
three of the four animals receiving the large dose of irradiated
ergosterol (Rats 40, 46, and 47) had a slightly lower ash content of
the femora than that of the animals which were killed at the be-
ginning of the experiment.   The femora of the rats which received
the smaller dose of vitamin D had an ash content of approximately
10 per cent more than that of the controls.   These animals were

on experiment but 15 days, which shows how very rapidly the percentage of ash in the long bones increases in a growing rat that is on a proper diet.

Although the percentage of ash in the femora of Rats 40, 46, and 47 was below that of the controls, it is no proof that the bones were demineralized. The total organic matter of a growing bone is continually increasing in absolute amount although its percentage of the total weight of the bone decreases. It may be possible that irradiated ergosterol in toxic amounts would have a greater inhibitory action on the calcification of the bone than it would have on the growth of the organic constituents of the bone. If this were the case the result would be a decrease in the percentage of ash but it would not be caused by any process of demineralization. It was thought that if such a condition did exist microscopic examination might show that the unossified areas in the bones of the rats receiving the irradiated ergosterol would be larger than those of the controls. To test this possibility three rats of a litter of six were killed at the age of 29 days. The other three members of the litter were given irradiated ergosterol (Table II, Rats 52 to 57). Instead of analyzing both femora of each animal for ash, the left femur in each case was preserved for histological examination and the right femur was analyzed for ash. As Table II shows, there was practically no difference between the ash content of the femora of the rats given irradiated ergosterol and the controls. Such being the case no marked difference in the histological picture could be expected, and this examination was not carried out.

Although there is an appreciable difference in individual cases between the ash content of the animals which received irradiated ergosterol and those which were killed as controls at the beginning of the experiment, the average difference between these two groups of animals, as Table II shows, is only a little more than 1 per cent.

The above data demonstrate that large doses of irradiated ergosterol have but a slight effect in reducing the percentage of ash in the bones of either adult or growing rats. This is decidedly different from the conclusions of the groups of investigators mentioned above. Furthermore, with growing animals the absolute amount of ash in the bone rather than its relative amount should be considered in determining whether irradiated ergosterol can withdraw

ash from bone. In Table III are given the weights of the young animals used in the experiments referred to above. The weights are given at the time the controls were killed and at the beginning

TABLE III

Influence of Large Doses of Irradiated Ergosterol upon Absolute Amount of Ash in Femora of Growing Rats

Rat No.	Weight of rat	Dose of ergosterol*	Weight of femur		Weight of femur ash	
			Right	Left	Right	Left
	gm.	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*
34	45	250	59.2	57.8	23.9	23.2
35	41	250	47.0	48.5	19.9	19.7
36	41	250	47.0	47.7	19.1	19.3
37	40	0	46.0	45.6	18.7	18.5
38	40	0	42.8	44.0	18.1	18.7
39	46	0	53.8	53.8	22.7	22.7
40	41	200	60.4	64.6	23.4	24.6
41	40	200	55.0	61.0	24.8	26.2
42	32	0	39.3	38.7	16.6	16.4
43	36	0	46.0	46.2	19.6	20.4
46	37	200	53.6	51.8	20.6	19.8
47	34	200	44.4	45.8	17.3	18.0
48	37	0	44.0	44.4	18.8	18.9
49	36	0	41.0	43.0	18.4	18.4
52	45	200	70.8		29.8	
53	49	200	77.7		34.0	
54	43	200	68.4 .		29.6	
55	42	0	52.2		22.8	
56	32	0	40.8		16.6	
57	47	0	60.8		25.5	

The average weight of the ash of the femora for the rats given irradiated ergosterol is 23.1 mg.; for the control rats is 19.5 mg.

    * Dose of irradiated ergosterol is expressed in cc. of the antirachitic equivalent of Steenbock standard cod liver oil.

of administration of irradiated ergosterol to the experimental animals. The weights of each femur analyzed and the weight of its ash are also given.

Although the difference in the amount of ash in the femora of

the experimental and control animals is small, Table III shows that there was slightly more ash in the femora of the animals which received irradiated ergosterol than in those which did not. The average difference per bone between the two groups is 3.6 mg. This difference expressed in mg. appears rather insignificant but it is 18 per cent of the weight of the ash of the bones of the control animals. The average weight of the bones of the experimental animals, is, however, sufficiently more than that of the controls to make the content of ash expressed in percentage of weight of bone less in the former than in the latter. The animals of the experimental and control groups weighed approximately the same. Thus the difference in weight of the bones must have been due to an increase in both inorganic and organic constituents of the bone of the experimental rats during the period of irradiated ergosterol administration. The increase of organic constituents was, however, more rapid than the increase of inorganic constituents, resulting in a decreased percentage of ash in spite of a slight increase in absolute amount.

The data presented here taken as a whole would seem to indicate that irradiated ergosterol even though given in extremely toxic doses has no specific action in withdrawing calcium salts from bone. These results are in accord with those of Harris (7) who has stated that by chemical analysis he found no loss of calcium or phosphorus from bone. Harris and Bills and Wirick (8) have shown that irradiated ergosterol is less toxic on a diet low in calcium than on a diet containing liberal amounts of this element. Jones, Rapoport, and Hodes (9) have also demonstrated that doses of irradiated ergosterol which will produce a marked hypercalcemia when there is calcium in the diet have only a slight effect on the level of blood calcium if a diet very low in calcium is fed. All these results indicate that vitamin D in large doses does not actually withdraw calcium from the bone. There are, however, so many factors to be considered that it is not presumed that it is absolutely impossible ever to bring about a decrease in the ash content of bones by administering large doses of irradiated ergosterol. Calcium salts are continually being absorbed from bone under normal conditions. This is especially true during the growing period. It is conceivable that this process of absorption might continue after the toxic action of irradiated ergosterol had pre-

vented the further deposition of calcium, or the rate of the absorption might even be increased by the action of large amounts of vitamin D.

A knowledge of the influence of large doses of irradiated ergosterol upon the balance of calcium in the animal body would be of value in determining whether or not the antirachitic factor in large quantities can withdraw appreciable amounts of calcium from bone. However, one of the first indications of the onset of vitamin D hypervitaminosis is anorexia, and in a short time the food consumption becomes negligible. The loss of appetite is accompanied by a pronounced decrease of body weight signifying rapid destruction of body tissue. Thus, with the intake of calcium reduced practically to zero and a simultaneous increased destruction of body tissue the obvious results would be a negative calcium balance. Consequently, decreased calcium retention does not necessarily show that vitamin D has any specific action in withdrawing calcium from the bone. In a recent investigation by Watchorn (10) the difficulties above mentioned have in part been overcome by using a diet very low in calcium. On this diet rats continued to eat and gain in weight although irradiated ergosterol was given in doses sufficient to produce loss of appetite when administered in conjunction with a diet containing calcium. None of the regular toxic effects of vitamin D hypervitaminosis were observed, but the irradiated ergosterol did produce a negative calcium balance. The amount lost per day was about 5 mg. per rat. In our experiments a loss of a few mg. of calcium per day, if taken from the entire skeleton, would not be detectable by ash analyses of individual bones. It is also possible that the calcium lost in the experiments of Watchorn was taken from tissues other than bone. This appears very tenable in view of the fact that there was no increase in excretion of phosphorus. It is, of course, conceivable that only the calcium of the bone which is bound with protein or the carbonate radical and not that bound with phosphate was released.

Regardless of the action irradiated ergosterol might have upon bone as a general systemic toxic substance the data presented here show that under the conditions of these experiments vitamin D in large toxic doses has no specific action in demineralizing the bone in any way comparable to its calcifying action when given in therapeutic amounts.

Pathological Report

Portions of the heart, aorta, kidney, stomach, small intestine, liver, and lung from twenty-nine experimental animals and six controls were fixed in Zenker's formol and sections stained with hematoxylin-eosin. The organs were not examined grossly and in each case only one section was prepared. All of the experimental animals showed the pathological lesions which have been described as characteristic of irradiated ergosterol intoxication. In most of the animals these lesions were of a severe grade. No lesions of a similar character were observed in the six control animals. The changes in the tissues of the experimental animals were briefly as follows:

Heart—Throughout the myocardium there are larger and smaller focal lesions composed of necrotic and calcified muscle fibers intermingled with and surrounded by a marked infiltration of large mononuclear cells. These lesions frequently are distinctly perivascular, and in most instances there is extensive degeneration and calcification of the arterial walls. Such lesions are present in the hearts of àll of the experimental animals, being of severe grade in sixteen and of moderate grade in thirteen.

Aorta—Fifteen of the animals show no change in their aortæ. In two the media is almost completely calcified. Five have extensive areas of medial calcification. Seven show medial degeneration with separation of the elastic fibers and a slight deposit of calcium in and about these fibers.

Kidneys—The most striking change is an extensive calcification which is usually most marked near the junction of the cortex and medulla. The calcium is deposited in the membrana propria of the tubules, in necrotic epithelial cells, and in the walls of small arteries. Occasionally the glomeruli are involved. Typical necrosis and calcification are present in the kidneys of all of the experimental animals, being of severe grade in twenty-one and of moderate grade in eight.

Gastrointestinal Tract—The lesions here consist of focal areas of necrosis and calcification occurring in the mucosa and in the muscularis. In the mucosa the calcium is deposited in epithelial cells. Calcified arteries are also encountered. The necrotic areas in the muscularis frequently are surrounded by monocytes. These le-

sions are present in twenty-three of the twenty-nine experimental animals, being of severe grade in nineteen and of moderate grade in four.

Lungs—No definite necrosis nor calcification is present.

Spleen—The spleens are congested and usually show a marked hemosiderosis. In one instance there is a massive area of necrosis with calcification.

Liver—No calcification occurs in the liver. Various grades of parenchymatous degeneration are the only constant finding.

SUMMARY AND CONCLUSIONS

The administration of toxic doses of irradiated ergosterol to adult rats does not produce any demonstrable demineralization of bone as determined by ash analyses of the femora.

The relative amount of ash in the femora of growing rats may be slightly decreased by large doses of irradiated ergosterol, but there is no detectable decrease in absolute amount.

Irradiated ergosterol in extremely toxic doses has no specific action which results in actually withdrawing calcium salts from bone comparable to its ossifying action when given in therapeutic doses.

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STUDIES ON THE SPONTANEOUS OXIDATION OF CYSTEINE

I. THE PREPARATION OF IRON-FREE CYSTINE AND CYSTEINE HYDROCHLORIDES*

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INTRODUCTION

In carrying out studies on the oxidation of cysteine, it is most important that cysteine be used which is known to be completely free from contaminating metals such as iron and copper, both of which are powerful catalysts to this type of oxidation. In a quantitative study of this action, Mathews and Walker (1) showed that this reaction was sensitive to iron in amounts as small as 0.00001 M concentration. This catalytic activity of traces of iron on cysteine oxidation is employed by Warburg (2) in a method devised to measure amounts of iron too small to be detected by the usual methods. Because of the ubiquitous nature of iron, and also of copper, the preparation of a metal-free cysteine has been extremely difficult.

The starting point is usually *l*-cystine, which is quite easily prepared from the keratins such as horn, feathers, hair, or wool by acid hydrolysis, with subsequent separation from the decolorized hydrolysate by careful neutralization. The cystine, which is obtained as typical hexagonal plates on recrystallization, is then reduced, usually by the tin and hydrochloric acid method described by Baumann (3) and by Friedmann (4). But cysteine

thus prepared even after several recrystallizations contains a considerable quantity of iron, most of which is an impurity of the tin used in the reduction. To remove this iron Sakuma (5) employed the combination of two methods which consisted essentially of the treatment of a cysteine solution with hydrogen sulfide and barium hydroxide. The iron is removed as the sulfide along with the barium hydrosulfide, and the residue after evaporation is extracted with alcohol. The cysteine hydrochloride thus removed is further purified by recrystallization from 5 volumes of ethyl or propyl alcohol. Cysteine so prepared is in the form of a mixture of the basic and neutral salt, the yield is very small, and there is always some danger of esterfication. Warburg (2) purifies his cysteine by pulverizing it as finely as possible in acetone in which cysteine hydrochloride is insoluble. The acetone has the property of taking up any iron chloride as well as other heavy metal chlorides. Cysteine thus prepared by him, however, still contained traces of iron.

<div align="center">EXPERIMENTAL</div>

Preparation of Iron-Free Cystine Hydrochloride—The *l*-cystine was prepared from human hair by the method described by Morrow (6). It was found during the preparation that cystine hydrochloride, being markedly insoluble in concentrated hydrochloric acid, may be readily purified from contaminating metals by precipitating it from its solution with hydrochloric acid gas. Because of the danger of contaminating the solution with traces of iron which might be dissolved from the softer types of glassware and even from Pyrex, quartz apparatus was employed throughout the steps of purification.

The cystine hydrochloride to be purified is dissolved by warming in the smallest possible quantity of pure distilled water contained in a quartz Erlenmeyer flask, which is then placed in a freezing mixture of ice and salt. Dry hydrochloric acid gas is then led into the flask through a quartz tube which contains a trap, the purpose of which is to prevent any condensed liquid which might carry impurities from the glass and.rubber of the gas generator from entering the solution. The tube is so arranged that the gas enters above the surface of the solution in the flask which must be gently shaken to facilitate absorption. At saturation, the

cystine hydrochloride precipitates in the form of prismatic needles which are then removed by filtration on a Buchner funnel fitted with acid-washed filter paper. The crystals are washed while on the filter paper with small amounts of pure concentrated hydrochloric acid which was specially prepared in quartz apparatus.

The mother liquor, which contains considerable amounts of cystine, was intensely yellow and when tested with KCNS solution gave a strong test for iron. As a matter of fact, during the recrystallizations the color of the mother liquor may serve as an indication of the presence of the metal. It has been found that iron in amounts as small as 1 part in a million has the property of imparting a distinct yellow color to a concentrated solution of hydrochloric acid (7). Cystine hydrochloride may be completely freed from iron by a sufficient number of recrystallizations carried out in this manner. If the cystine hydrochloride is to be used in the preparation of cysteine, it will be unnecessary to recrystallize more than twice since traces of iron will be added later during the reduction as impurities of the tin.

Reduction of Cystine—If a reducing substance could be found which would reduce cystine quantitatively and which could be obtained in an iron-free state, the preparation of pure cysteine hydrochloride would be comparatively simple. A reducing gas naturally suggests itself. Attempts to reduce cystine by means of sulfur dioxide resulted in incomplete reduction which had been noted by Andrews (8) who followed the degree of reduction by polarimetric measurements. In the same study, he found that sulfides and sulfites likewise produced incomplete reduction. Since potassium cyanide is employed as the reducing agent in Sullivan's naphthoquinone test for cysteine (9), an attempt was made to reduce cystine by means of hydrocyanic acid gas in a medium made slightly alkaline with pure ammonium hydroxide. This, too, resulted in an incomplete reduction with the formation of an unknown polymerized cyanide decomposition product which formed a dark resinous mass on concentration.

Undoubtedly the most efficient method of reduction is that which makes use of tin and hydrochloric acid. The objection to this method arises from the possibility of contamination of the preparation by iron from the tin, as it is impossible to obtain tin

in a state free from this metal.   The solution lies in an efficient
and complete purification of cysteine thus prepared.

Preparation of Iron-Free Cysteine Hydrochloride—Even though
cysteine hydrochloride is considerably more soluble in concen-
trated hydrochloric acid than is the corresponding cystine salt,
yet it can be precipitated by absorption of hydrochloric acid gas
from its strongly cooled solution.   The simplicity of the process
and completeness of purification justify the method used.   The
cysteine hydrochloride was repeatedly recrystallized by the method
described for the preparation of the iron-free cystine salt.   The
purification was carried out exclusively in fused quartz vessels
which were repeatedly boiled out with strong hydrochloric acid,
and were proved to be iron-free by the method described below.

To prove that the reagents and method did not add any iron
to the preparation, the following preliminary experiment was
performed.   Water was distilled from a quartz distilling flask
into a quartz Erlenmeyer flask cooled by a stream of water.   Pure
concentrated hydrochloric acid was then boiled in the distilling
flask, the side tube of the latter being placed just above the level
of the water in the Erlenmeyer flask.   The distillation was con-
tinued until the resulting acid solution in the receiver was 6 N
as determined by titration.   50 cc. of the acid were then evapo-
rated to dryness in a quartz evaporating dish over an aluminum
sand bath carefully protected from dust by an especially con-
structed glass hood.   The evaporation was carried out in the
presence of a small crystal of pure $KClO_3$, the purpose of which
was to oxidize any ferrous iron to the ferric state.   To the residue
were then added 1 cc. of the distilled acid and 1 cc. of pure dis-
tilled water.   This acid solution was carefully washed about the
sides of the vessel and then poured into a clear acid-washed test-
tube, to which was added 1 cc. of a 10 per cent solution of KCNS
specially purified by recrystallization.   The tube was then viewed
over a perfectly white background and compared with one used
as a control and which contained 3 cc. of the reagents.   The final
concentration of the acid was 2 N, at which the test is most sen-
sitive according to Lachs and Friedenthal (10).

An absence of color indicates that the 50 cc. of acid solution
contained less than 0.0000001 gm. of iron, which is the limit of
sensitivity.   The water and acid solutions used in the purification

were freshly distilled in quartz in the manner described above and stored in 500 cc. quartz flasks equipped with quartz ground stoppers, the tops of which were kept at all times covered with cellophane to insure freedom from dust. Amounts of acid and water prepared in this manner when evaporated in quantities as large as 500 cc. and the theoretical residues tested failed to give any test for iron.

After this preliminary test the cysteine hydrochloride solution replaced the water in the quartz Erlenmeyer flask, cooled by salt and ice, and hydrochloric acid was distilled into it until the cysteine hydrochloride was precipitated. The crystals were filtered with every precaution to avoid contamination with iron, redissolved, and reprecipitated.

The crystallizations were repeated until 10 cc. of the mother liquor, when evaporated and ignited in a quartz crucible and tested in the above manner, failed to give a test for iron. The number of recrystallizations necessary to remove all traces of iron naturally depends upon the quantity of iron originally present in the crude cysteine hydrochloride. For an especially pure sample five or six crystallizations are sufficient, while samples less pure will require more, but since the purification does not involve spontaneous crystallizations, which require standing over a considerable number of hours or even days, a large number of crystallizations may be carried out in several hours.

A 2 gm. sample of cysteine hydrochloride purified in this manner was ignited and when tested by the KCNS method gave not the slightest trace of color, indicating that a 20 mg. sample, the quantity commonly used in the ordinary oxidation experiment in the Barcroft apparatus, contained less than 0.000000001 gm. of iron. Small traces of copper which may have been present in the crude preparation were completely removed long before the iron in the early crystallizations. The purification involves a loss of 50 to 90 per cent.

A sample of cysteine so prepared possessed a specific rotation of $[\alpha]_D^{26°} = +6.16°$. When determined a year later after storage as cysteine hydrochloride in a desiccator charged with $CaCl_2$ and containing an open beaker holding a small quantity of concentrated hydrochloric acid, the same sample possessed an optical activity of $[\alpha]_D^{26°} = +6.05°$. This emphasizes the stability of

the purified dry cysteine hydrochloride even when exposed to oxygen. This is contrary to views expressed by Andrews (8) who states that the most elaborate precautions to exclude oxygen are necessary to prevent oxidation. Exposure of the dry cysteine hydrochloride to ultra-violet light over periods of 10 to 20 hours, which, it was believed, might hasten oxidation, likewise failed to bring about any change in optical activity.

SUMMARY

A method for the preparation of iron-free cysteine and iron-free cystine is described by which it is possible to prepare cysteine hydrochloride containing no detectable traces of iron and certainly less than 1 part of iron in 20 million parts of cysteine hydrochloride.

Cysteine hydrochloride thus prepared is stable on exposure to oxygen when stored in the dry state.

Such cysteine hydrochloride free from iron was used to test Warburg's theory that the spontaneous oxidation of cysteine depends upon iron. The results will be given in a later paper, proving that this oxidation occurs at a slow rate in the absence of iron.

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HYDROGEN ION CONCENTRATION AND ACID-BASE EQUILIBRIUM IN NORMAL PREGNANCY

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INTRODUCTION

In 1912 Hasselbalch, with Lundsgaard and Gammeltoft (6, 7), described a lowering of the alveolar carbon dioxide and of the carbon dioxide-combining power of the blood in the later months of normal pregnancy. These changes have been abundantly confirmed by others and found to appear early in the course of pregnancy. These findings together with an increased ammonia to total nitrogen ratio in the urine described by Hasselbalch and Gammeltoft (6) have long been considered as an expression of an increased acid production in pregnancy.

This conception has arisen partly from the impression that Hasselbalch and his associates detected a reduction in the pH of the blood in pregnancy. In actual point of fact they did not determine the pH of the blood directly, but only after exposure to a known concentration of CO_2 in air. This is equivalent, of course, to measuring the bicarbonate content or alkaline reserve and yields no information concerning the actual reaction of the blood in the body. The distinction was clearly recognized by Hasselbalch who concluded that the hydrion concentration of the blood in pregnancy remained normal.

Hasselbalch did, however, interpret the increased ammonia to nitrogen ratio as an indication of an abnormally great acid production. It is now recognized that acid production is proportional not to the urinary ammonia to nitrogen ratio, but to the ab-

solute quantity of ammonia in the urine. The latter does not appear to be unusually large during pregnancy (13).

The conception that the reduced alkali reserve might be due not to increased acid products, but to a base deficit, was advanced by Marrack and Boone (8) in 1923, because of their failure to find increased cations which they believed should be present if there were a demonstrable increase in the anions. Oard and Peters (9) described the total acid-base equilibrium of the blood serum in pregnancy. They found that the reduced carbon dioxide content and lowered serum proteins were accompanied by a comparable reduction of total base and that there was no increase in the un-determined acids of the blood.

These studies indicate that the reduced alkali reserve is due to a reduction of the cations and that there is no "acidosis of preg-nancy." Williamson (16) in 1923 reports the pH normal in five cases, although he does not state the method. Marrack and Boone (8) determined the pH colorimetrically and found a shift from the normal toward alkalinity (normal 7.30 to 7.45 pH, pregnant 7.25 to 7.55 pH).

Because of the unsatisfactory status of the value of pH in preg-nancy, it was thought desirable to extend the investigation of the total acid-base equilibrium to include determination of pH. While these investigations were in progress Stander, Eastman, Harrison, and Cadden (14) reported three electrometric pH de-terminations which lie within their determined normal range.

Methods

The blood for these studies was withdrawn from the antecubital vein anaerobically and without stasis (or with only a minimal preliminary amount of stasis in those cases in which the veins were not otherwise apparent) because of the inspissating effect of stasis (10, 12). The blood was handled and the serum separated according to the anaerobic technique of Austin et al. (1).

The hydrogen ion concentration was determined according to the gasometric method of Eisenman (5). However, by utilizing Van Slyke and Neill's micro method for the CO_2 analyses (15) the procedure was carried out with less than 5 cc. of serum. 2 cc. of serum were introduced into each of two tonometers, one hav-ing a CO_2 partial pressure of 30 mm. and the other of 60 mm.

These were saturated for 20 minutes and each serum was then analyzed in duplicate for CO_2 content, as was the original serum. The value $pK'_1 = 6.12$ was used in our calculations. The total proteins and protein fractions were determined by the modification of Howe's technique described by Bruckman, D'Esopo, and Peters (3). In order to conserve serum a few of the total proteins were determined by a micro modification. 0.2 cc. of serum was diluted with 2.5 cc. of physiological saline solution. 1 cc. aliquots were digested by the micro-Kjeldahl

TABLE I

Serum Electrolytes and pH in Pregnant and Non-Pregnant Women

	Protein	Albumin	Globulin	CO₂	pCO₂	pH	Total base
	per cent	per cent	per cent	vols. per cent	mm.		mм
Pregnant individuals							
No. of patients	22	10	10	22	10	10	19
Maximum	6.95	4.69	3.58	59.2	42.6	7.41	155.3
Minimum	5.39	2.75	1.71	46.0	37.6	7.28	139.1
Average	6.21	3.53	2.69	52.6	40.3	7.36	145.8
Non-pregnant individuals							
No. of observations	27*	27*	27*	8	8	8	10†
Maximum	7.65	5.65	2.91	65.4	51.9	7.48	158.0
Minimum	6.46	4.37	1.32	56.3	31.1	7.33	152.5
Average	6.93	5.06	1.89	61.6	44.1	7.39	153.8

* Bruckman, D'Esopo, and Peters (3).
† Oard and Peters (9).

technique and distilled into 0.02 N acid. The non-protein nitrogen was determined in a trichloroacetic acid filtrate by the method of Bock and Benedict (2). The remaining electrolytes were determined by methods described by Oard and Peters (9).

Results

Summarized in Table I are the results of Oard and Peters (9) to which have been added the data collected in the present study. Chloride, phosphorus, and undetermined acid values have been

omitted as they do not differ materially from normal non-pregnant values. In order to establish a comparable normal standard, hydrogen ion determinations were carried out on eight normal non-pregnant individuals by a technique as nearly similar as possible to that used on gravid individuals. These results are summarized in Table I. The remaining determinations on non-pregnant inviduals were performed by techniques comparable to those used in the present study and have been previously reported.
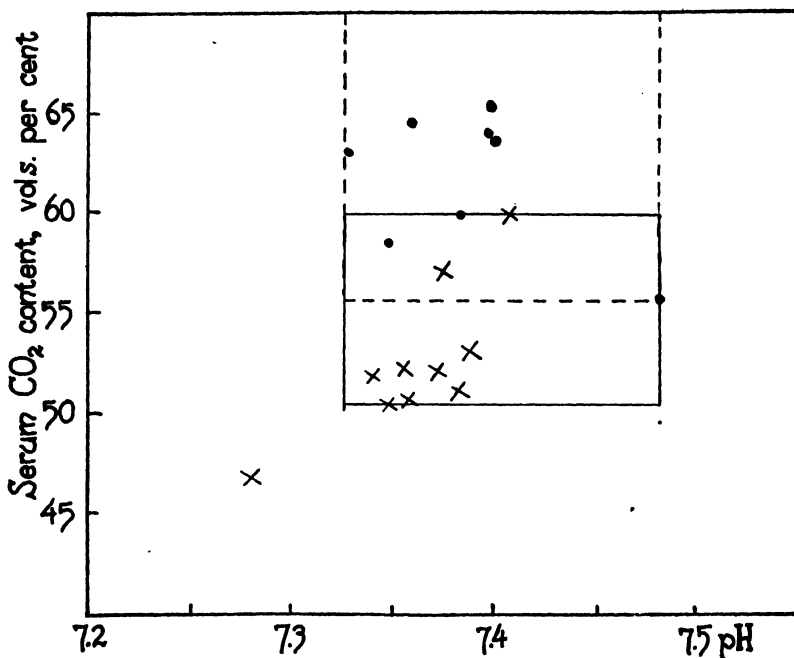
Chart 1. The CO_2 content and pH of the serum in normal and pregnant women. Values obtained in pregnancy are represented by crosses, in the non-pregnant by dots. The broken lines delimit the non-pregnant values and the solid lines the values in pregnancy.

The results indicate that in normal pregnant individuals the carbon dioxide content of the serum is reduced from the normal by about 9 volumes per cent, but the partial pressure of CO_2 is about 4 mm. lower than normal. By virtue of the reduced CO_2 tension the pH in gravid individuals coincides with that in the non-pregnant. This is shown graphically in Chart 1. The results of one

determination that fall outside this range were from serum obtained from an individual who, although normal according to our criteria, had, during a previous pregnancy, developed a severe unexplained anemia.

The lowering of the serum total proteins has long been recognized and it is also well established that fibrinogen is increased during the course of normal pregnancy. Concerning the fractions, albumin and globulin, however, Plass and Mathews (11) in reviewing the literature found considerable confusion. These authors found a definite decrease in the albumin fraction and a relative increase in the globulin fraction when compared to their normal figures. This has been confirmed by Eastman (4) and the present study.

Seven further observations on total base in pregnancy are added. These are in full agreement with the values previously reported (9, 14).

SUMMARY

In normal pregnancy the bicarbonate of serum falls without change in the hydrogen ion concentration. The serum proteins are reduced at the expense of the albumin fraction, the globulin being at the normal level or relatively slightly increased. The values for serum chloride, phosphorus, and undetermined acids are the same as in normal non-pregnant individuals. The reduction in the anions is balanced by a reduction of total base.

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DIETARY REQUIREMENTS FOR FERTILITY AND LACTATION

XXIII. THE SPECIFIC EFFECT OF VITAMIN B ON LACTATION*

By BARNETT SURE and DOROTHY J. WALKER

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In previous publications (1) records were presented showing that lactating rats given diets deficient in vitamin B consume approximately 50 per cent less of food daily than nursing mothers receiving the same rations fortified with an abundance of this vitamin. It was, therefore, tentatively concluded that the reduction of the plane of nutrition was the determining factor responsible for failure of nursing young on the above dietary regimen. In order to subject this hypothesis to a critical examination, the paired-feeding type method of experimentation was adopted (2).

Briefly stated, the technique used was as follows: Female rats were transferred on the day of the birth of their litters, which were reduced to six in number, from our Stock Diet 1 (3) to the following experimental ration: casein,[1] 20; Salt Mixture 185 (4), 4; autoclaved yeast, 10; butter fat, 10; dextrin, 56. Litter mates were allowed the same ration, but the autoclaved yeast was replaced by the same proportions of untreated yeast[2] in the diet, and the mothers were restricted to the same daily amounts of food and water intake as were consumed by the lactating rats receiving the vitamin B-deficient ration. With the plane of nutrition kept constant, any improvement in the lactation performance of the latter

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* Research paper No. 201, Journal Series, University of Arkansas.

[1] Purified by extraction for 10 days with acidulated water.

[2] Northwestern Yeast Company dehydrated yeast was used. The autoclaving of the yeast was performed in shallow glass Pyrex dishes for 6 hours at 20 pounds pressure.
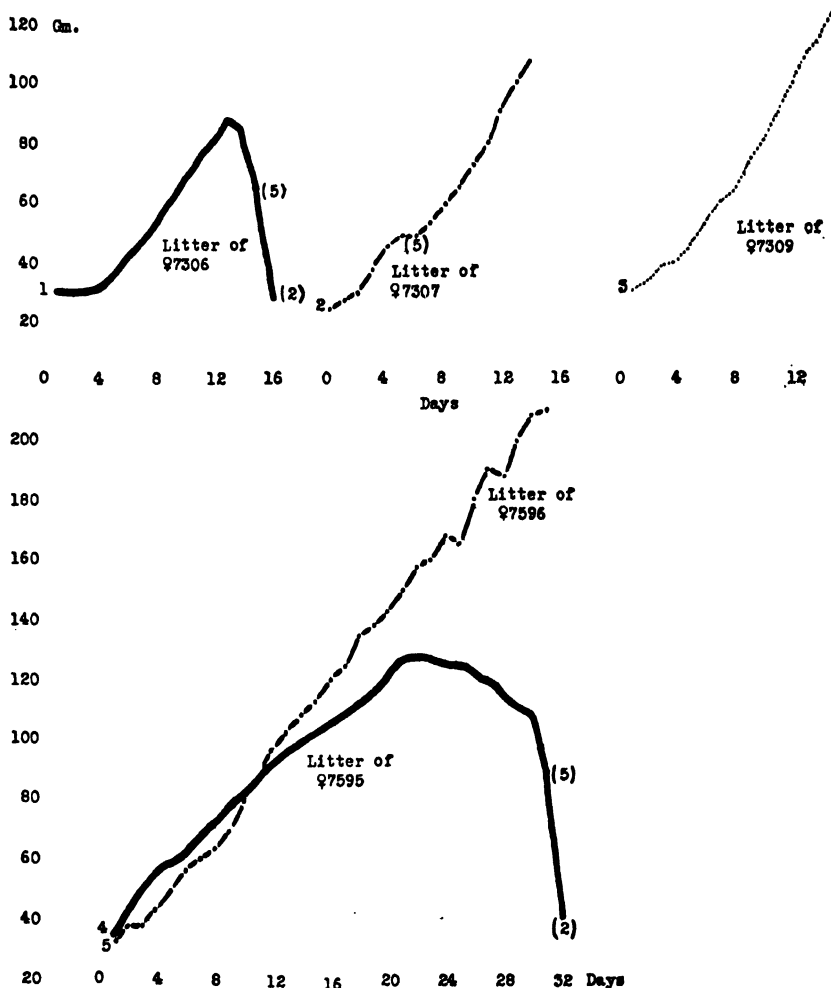
CHART I. Curve 1 represents the lactation record of Female 7306, the diet of which was deficient in vitamin B; Curve 2, the lactation record of Female 7307, on the same diet containing an abundance of vitamin B. This lactating rat was restricted to the same daily food and water intake as that consumed by its litter mate, Female 7306. Curve 3 represents the lactating record of Female 7309, also a litter mate, which is a positive control; in other words, this lactating female was allowed the same diet given Female 7307 containing an abundance of vitamin B, but was not restricted to its food and water intake. Curve 4 represents the lactation record of Female 7595, whose diet was deficient in vitamin B; Curve 5, the lactation

group must necessarily be credited to the specific influence of vitamin B. Such was actually the case in all the paired-feeding trials performed. In order, however, to determine the influence of inanition, which is an accompanying symptom complex in vitamin B deficiency during lactation as well as growth, a number of positive controls were studied, which were given the above synthetic diet containing 10 per cent untreated dehydrated yeast, and were not restricted to the daily food and water intake. Our results are summarized in Charts I to III.

On Chart I it will be noted that Female 7307 received the same diet and same daily food and water as its litter mate, Female 7306, the only difference being that the ration contained in addition an abundance of vitamin B. On the 15th day of lactation Female 7307 was still rearing its litter of five young out of six, weighing collectively 109 gm., while Female 7306, on the same plane of nutrition, but on a vitamin B-deficient ration, had at that period encountered considerable infant mortality, having reduced its litter of six to two, which were in an emaciated dying condition when the experiment was terminated. The specific influence of vitamin B is, then, very pronounced. Female 7309 served as a positive control. This lactating animal received the same diet as its litter mate, Female 7307, but was allowed food and water *ad libitum*. On the 15th day its litter collectively weighed 125 gm. The inanition effect is, therefore, represented by the difference in the collective weight of the litter of Female 7309 and that of Female 7307, which is 16 gm.

The specific effect of vitamin B on lactation is even more pronounced in the case of litters of Females 7595 and 7596, represented in Curves 4 and 5. The prolongation of the lactation period of

record of Female 7596, which was given the same diet but fortified with an abundance of vitamin ·B, and was restricted to the same daily food and water intake as that consumed by its litter mate, Female 7595.

10 per cent Northwestern yeast in the diet supplied adequate amounts of the vitamin B complex for successful lactation. The females whose rations were deficient in vitamin B received in their diets 10 per cent Northwestern yeast, autoclaved for 6 hours at 20 pounds pressure. This yeast provided an abundance of vitamin G for lactation, so that uncomplicated vitamin B deficiency was produced in the nursing young. Figures in parentheses represent the number of young left on the date indicated.

Female 7595 can be explained on the basis of greater storage of vitamin B. This gave us an opportunity of studying the lactation performance of a litter mate on the reduced plane of nutrition, which takes place during this avitaminosis over a longer nursing
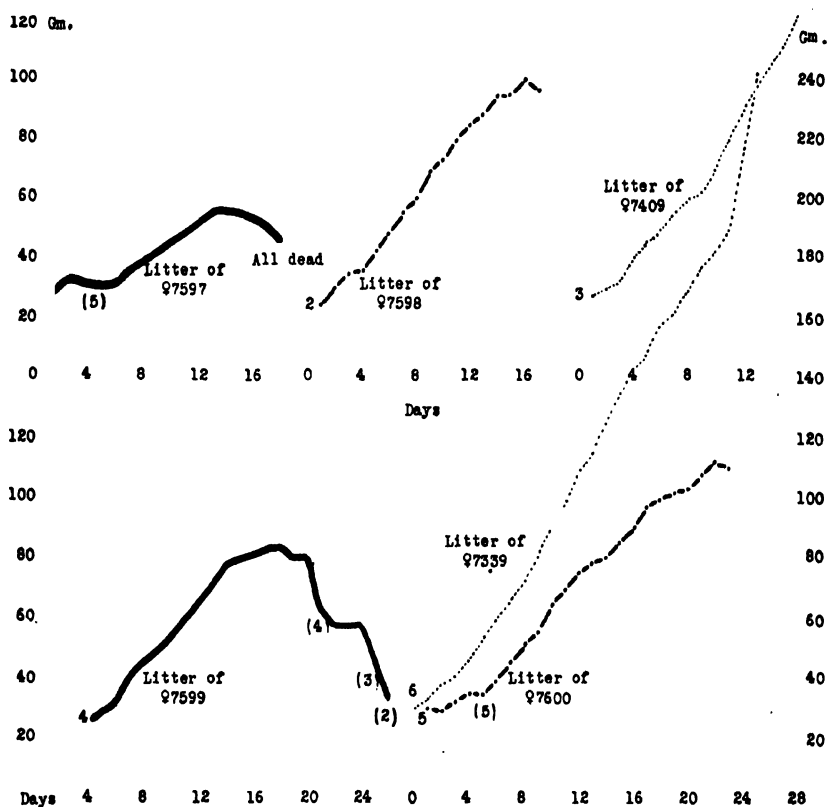
CHART II. Curves 1 and 4 represent the lactation records of Females 7597 and 7599 whose diets were deficient in vitamin B. Curves 2 and 5 represent the lactation records of Females 7598 and 7600 which were given the same diet, containing an abundance of vitamin B, but were restricted to the same daily amounts of food and water as were consumed by their litter mates, Females 7597 and 7599. Curves 3 and 6 represent the lactation records of Females 7409 and 7339, which are positive controls. These females received the same diet as Females 7598 and 7600, but were unrestricted in the daily food and water intake. Figures in parentheses represent number of young left on the date indicated.

CHART III. Curves 1, 3, and 5 represent the lactation records of Females 7601, 7603, and 7605 whose diet was deficient in vitamin B. Curves 2, 4, and 6 represent the lactation records of Females 7602, 7604, and 7606, which were given the same diet but fortified with an abundance of vitamin B, and were restricted to the same daily amounts of food and water as were consumed by their litter mates, Females 7601, 7603, and 7605. Figures in parentheses represent number of young left on the date indicated.

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period. The specific influence of vitamin B on growth of the nursing young became apparent only after the 11th day. From then on the litter of Female 7595, receiving the vitamin B-deficient ration, gradually began to grow slowly, then declined, and finally collapsed on the 32nd day of lactation, four young out of six having died at that time. During the entire lactation period the litter of Female 7596, on the same food and water intake, and on the same maternal diet containing an abundance of vitamin B, made appreciable and continuous gains in weight, and on the 31st day of lactation was in excellent state of nutrition, and collectively weighed 210 gm.

Curves 1, 2, 4, and 5 of Chart II furnish additional evidence which conclusively demonstrates the specific effect of vitamin B on lactation. During a period in lactation when the infant mortality on the maternal diet, deficient in vitamin B, was 70 to 100 per cent, litter mate females on the same plane of nutrition, and receiving the same diet containing an abundance of vitamin B, were still rearing their entire litters of six to a collective weight of 100 to 120 gm. The same picture is also apparent from Curves 1 to 6 inclusive, submitted in Chart III.

The effect of a restricted food and water intake on lactation is best illustrated by a comparison of the lactation records of Female 7600, which was restricted, and Female 7339 (Curves 5 and 6, Chart II), which was not restricted in food and water intake. Both animals received the same ration containing adequate amounts of vitamin B for lactation. The inanition effect is represented by the difference between the collective weight of the litter of Female 7339 and that of Female 7600, which is 132 gm.

It is quite evident, then, from the data presented that vitamin B, in addition to being an influencing factor in controlling the plane of nutrition, also exerts a specific effect on lactation, characterized by the growth and survival of nursing young, unrelated to food and water intake.

We are at present finding similar results on the specific effect of vitamin B on the growth of non-lactating animals.

SUMMARY

Vitamin B, in addition to influencing food consumption, plays a specific rôle in lactation, unrelated to the plane of nutrition.

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ON WALDEN INVERSION

XV. THE INFLUENCE OF SUBSTITUTING GROUPS ON OPTICAL ROTATION IN THE SERIES OF DISUBSTITUTED PROPIONIC ACIDS CONTAINING A METHYL GROUP

By P. A. LEVENE and R. E. MARKER

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In a previous communication Levene and Mikeska[1] reported on the changes in the rotations of the derivatives of 2-substituted propionic acid (3) (disubstituted acetic) produced by changing the character of the functional groups. The primary aim of the investigation was to trace the effect of the substitution of the hydroxyl by a halogen in the disubstituted ethanols. In earlier communications, Levene and Mikeska[2] arrived at the conclusion that in optically active secondary carbinols, the substitution of the hydroxyl by a halogen results in a change of direction of rotation. This view has been recently criticized by Houssa, Kenyon, and Phillips.[3] The conclusion of these authors is based on indirect evidence as was also the conclusion of Levene and Mikeska. The decision between the two views will depend upon further data. Some such data are contained in the present article. We must, however, state that the present work was undertaken not because of the criticism of the above writers but for the reason that the 2-substituted propionic acids proved unsatisfactory material because of the racemization which occurred in the series of reactions that had been planned. The assumed mechanism of the racemization was

[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84**, 571 (1929).
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discussed in the paper by Levene and Mikeska and if their reasoning is correct, then the series of disubstituted propionic acids should not present the same difficulty. In reality, such was the case. This series, however, presented greater difficulty in the resolution of the higher members.

Comparing the effect of the substituents on rotation, it is noted from Tables I, II, and III that it is practically identical in the four

TABLE I

Molecular Rotations of Derivatives of Alkyl 2-Substituted Butyric Acid in Homogeneous State ($[M]_D^{25° \pm 3°}$)

	$-\overset{\text{O}}{\underset{|}{\overset{\|}{C}}}-OH$	$-\overset{\text{O}}{\underset{|}{\overset{\|}{C}}}-OC_2H_5$	$-CH_2OH$	$-CH_2Br$	$-CH_2Cl$
$C_2H_5\!-\!CH\!-\!CH_2\!-$ $\quad\quad\ \ \|$ $\quad\quad\ CH_3$	+4.21	+4.69	+3.69		+8.95
$n\!-\!C_3H_7\!-\!CH\!-\!CH_2\!-$ $\quad\quad\quad\ \ \|$ $\quad\quad\quad\ CH_3$	+2.84 (+3.28)*	+0.55 (0.66)*	+1.62 (+1.91)*	−16.81	 (−4.93)*
$n\!-\!C_4H_9\!-\!CH\!-\!CH_2\!-$ $\quad\quad\quad\ \ \|$ $\quad\quad\quad\ CH_3$	+5.45	+2.61	+3.58	−15.03	
$n\!-\!C_5H_{11}\!-\!CH\!-\!CH_2\!-$ $\quad\quad\quad\ \ \ \|$ $\quad\quad\quad\ \ CH_3$	+7.13	+3.74	+5.39	−13.02	
$n\!-\!C_6H_{13}\!-\!CH\!-\!CH_2\!-$ $\quad\quad\quad\ \ \ \|$ $\quad\quad\quad\ \ CH_3$	+1.32	+0.75	+0.67	−1.56	

* This series was duplicated starting from acids of different rotations.

higher members, being for the dextrorotatory series in the following descending order.
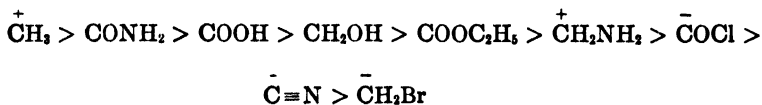
$$\overset{+}{C}H_3 > CONH_2 > COOH > CH_2OH > COOC_2H_5 > \overset{+}{C}H_2NH_2 > \overset{-}{C}OCl >$$

$$\overset{-}{C}\!\equiv\!N > \overset{-}{C}H_2Br$$

TABLE II
Molecular Rotations of Derivatives of Alkyl 2-Substituted Butyric Acid in Homogeneous State ($[M]_D^{24° \pm 3°}$)

	—C(=O)—OH	—C(=O)—Cl	—C(=O)—NH$_2$*	—CN	—CH$_2$NH$_2$
C$_2$H$_5$—CH—CH$_2$— | CH$_3$	+4.21	+7.33	+2.32	+3.41	+4.31
n—C$_3$H$_7$—CH—CH$_2$— | CH$_3$	+2.84	−3.65	+5.36	−3.64	+0.29
n—C$_4$H$_9$—CH—CH$_2$— | CH$_3$	+5.45	−3.16	+8.85	−5.41	+1.73
n—C$_5$H$_{11}$—CH—CH$_2$— | CH$_3$	+7.13	−2.40	+10.49	−5.59	+3.17

* The rotations of the amides were taken in 75 per cent alcohol.

TABLE III
*Molecular Rotations of Carbinols, Bromides, and Hydrocarbons**

	—CH$_2$OH	—CH$_2$Br	—CH$_2$·CH$_2$OH	—CH$_2$·CH$_2$Br	—CH$_2$·CH$_3$	—CH$_3$
C$_2$H$_5$—CH—CH$_2$— | CH$_3$	+1.70	+5.21	+2.25	+4.22	+1.67	
n—C$_3$H$_7$—CH—CH$_2$— | CH$_3$	+1.40†	−16.81				+7.75
n—C$_4$H$_9$—CH—CH$_2$— | CH$_3$	+3.58†	−15.19	+0.65	−7.51	+1.36	
n—C$_5$H$_{11}$—CH—CH$_2$— | CH$_3$	+5.39†	−13.02	+2.29	−5.57		+10.96

* $[M]_D$ was taken at an average temperature of 25°.

† This carbinol and the derivatives given in this table were levorotatory but were tabulated as dextrorotatory for convenience of discussion.

In the 2-propylbutyric acid (4) the $COCl$ and $C \equiv N$ have practically the identical rotation and their order may be interchanged. In detail the order of change is different from that observed in the series described by Levene and Mikeska.   In the two most significant derivatives, the carbinol and the halide, the directions of the rotations are different in the old and in the new series; whereas in the 2-substituted propionic acids, the dextro acids lead to a levo-carbinol and a dextro-halide, the dextro-2-substituted butyric acids lead to a dextro-carbinol and to a levo-halide, except in the case of the 2-ethylbutric acid (4), which leads to a dextro-carbinol and to a dextro-halide.   Furthermore, in this case the numerical value of the rotation of the halide is higher than that of the carbinol.   Thus on passing from carbinol to halide in this case, the change in rotation is in a direction opposite to that of the change in the other corresponding members of this series.   In a general way, then, with the exception of the one case, *the configurationally related carbinols and halides rotate in opposite directions, this being the relationship which Levene and Mikeska have assumed for the secondary carbinols and for the corresponding halides.*

Case of 2-Ethylbutyric Acid (4) (3,3-Methylethylpropionic) Series—The exceptional position of the members of this series *a priori* seemed to be possible to explain on the assumption that they are configurationally enantiomorphous to the dextrorotatory higher members in the following manner.

$$CH_3$$
$$|$$
$$C_2H_5-C-CH_2COOH \qquad COOH-CH_2-C-C_3H_7$$
$$|$$
$$H \qquad\qquad\qquad\qquad H$$

Dextro (levo)   Levo (dextro)
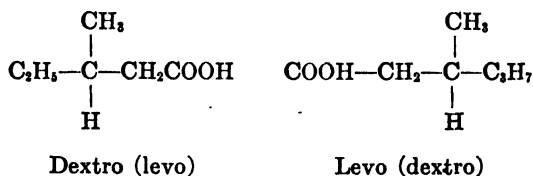
If this were so, then the change to the right from the dextro-carbinol to the higher dextro-halide would be a change in the same direction as that of the levo-carbinols of the higher homologues to their dextro-halides.   Because of these considerations, it was decided to correlate the configurations of dextro-2-ethyl- and dextro-2-propylbutyric acids (4).

It is evident that if the acids are of the same configuration, then by converting the $-CH_2COOH$ of the first into C_3H_7 and that of

the second into C_2H_5 two enantiomorphous hydrocarbons (methyl-ethylpropylmethanes) should result.

$$
\begin{array}{cccc}
\overset{\displaystyle CH_3}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C_2H_5-C-CH_2COOH}}}} \rightarrow
\overset{\displaystyle CH_3}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C_2H_5-C-C_3H_7}}}} \quad
\overset{\displaystyle CH_3}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C_3H_7-C-C_2H_5}}}} \leftarrow
\overset{\displaystyle CH_3}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C_3H_7-C-CH_2COOH}}}} \\
\quad I \qquad\qquad\qquad II \qquad\qquad\quad III \qquad\qquad\qquad IV
\end{array}
$$
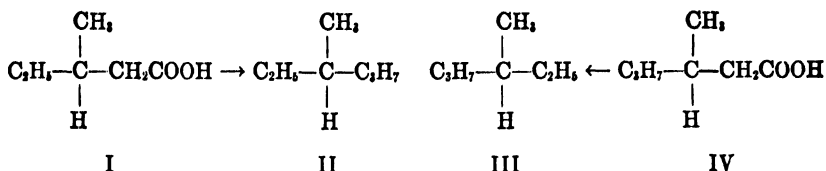
On the other hand, if the acids are enantiomorphously related, both should lead to the identical hydrocarbon.

$$
\overset{\displaystyle CH_3}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C_2H_5-C-CH_2COOH}}}} \rightarrow
\overset{\displaystyle CH_3}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C_2H_5-C-C_3H_7}}}} \leftarrow
\overset{\displaystyle CH_3}{\underset{\displaystyle H}{\overset{|}{\underset{|}{HOOC-CH_2-C-C_3H_7}}}}
$$
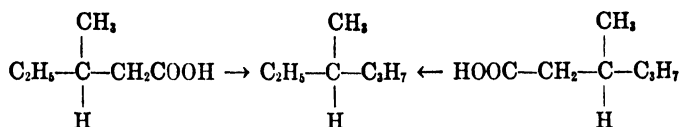
It was found that both acids yielded a dextrorotatory hydrocarbon. Hence, *the dextrorotatory 2-ethylbutyric acid (4) and the dextrorotatory 2-propylbutyric acid (4) are of opposite configurations.* From this observation it also follows that the *configurations of the 2-substituted butyric acids (4) and their derivatives can be correlated on the basis of the changes in the direction of the rotations in passing from the carbinols to the halides.* The set of reactions which lead from 2-ethylbutyric acid (4) to the hydrocarbon is the following.

$$
\underset{\displaystyle C_2H_5}{\overset{\displaystyle CH_3}{\diagdown\,\diagup}}CH\,CH_2COOH \rightarrow
\underset{\displaystyle C_2H_5}{\overset{\displaystyle CH_3}{\diagdown\,\diagup}}CHCH_2CH_2OH \rightarrow
\underset{\displaystyle C_2H_5}{\overset{\displaystyle CH_3}{\diagdown\,\diagup}}CHCH_2CH_2Br \rightarrow
$$

$$
\underset{\displaystyle C_2H_5}{\overset{\displaystyle CH_3}{\diagdown\,\diagup}}CHCH_2CH_2MgBr + HCHO \rightarrow
\underset{\displaystyle C_2H_5}{\overset{\displaystyle CH_3}{\diagdown\,\diagup}}CHCH_2CH_2CH_2OH \rightarrow
$$

$$
\underset{\displaystyle C_2H_5}{\overset{\displaystyle CH_3}{\diagdown\,\diagup}}CHCH_2CH_2CH_3Br \rightarrow
\underset{\displaystyle C_2H_5}{\overset{\displaystyle CH_3}{\diagdown\,\diagup}}CHC_3H_7
$$
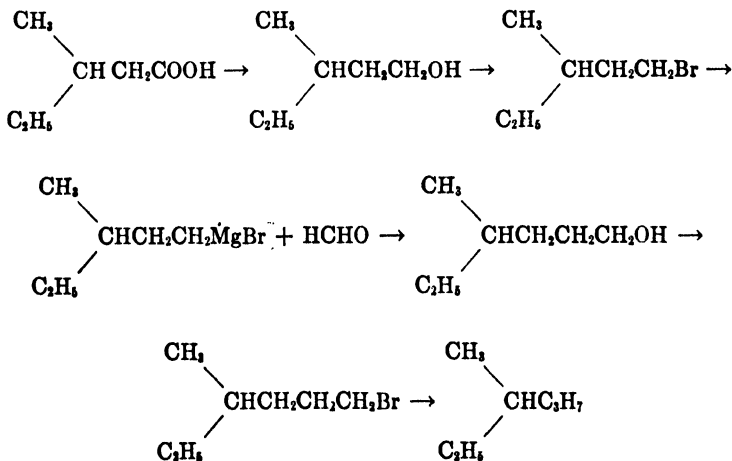
In this place it must be recalled that the same dextrorotatory hydrocarbon which was obtained in our case from the 1-brom-3-methyl hexane had been obtained many years ago by Marckwald[4] from dextro-2-ethylpropyl (active amyl) iodide (3) by condensation with ethyl iodide. The dextro-iodide employed by him is a derivative of the levo-amyl alcohol, which in its turn, can be converted into dextro-valeric acid. Thus, through the reactions reported by us and those of Marckwald, levo-amyl alcohol and the dextro-2-ethylbutanol (4) and dextro-2-ethyl pentanol (5) and the corresponding dextro-halides are all configurationally related. In addition through the work of Van Romburgh[5] and of Walden[6] the dextro-2-ethylbutyric acid (4) and 2-ethylvaleric acid (5) are correlated with 2-ethylpropionic acid (3) (active valeric). These relationships are given in the following formulæ.

$$
\begin{array}{ccc}
\text{COOH} & \text{CH}_2\text{OH} & \text{CH}_2\text{Br} \\
| & | & | \\
\text{H—C—C}_2\text{H}_5 \rightarrow & \text{H—C—C}_2\text{H}_5 \rightarrow & \text{H—C—C}_2\text{H}_5 \text{————————} \\
| & | & | \\
\text{CH}_3 & \text{CH}_3 & \text{CH}_3
\end{array}
$$

Dextro + 18.21   Levo − 5.19   Dextro + 5.56

$$
\begin{array}{cccc}
\text{CH}_2\text{COOH} & \text{CH}_2\text{CH}_2\text{OH} & \text{CH}_2\text{CH}_2\text{Br} & \downarrow \\
| & | & | & \text{C}_3\text{H}_7 \\
\text{H—C—C}_2\text{H}_5 \rightarrow & \text{H—C—C}_2\text{H}_5 \rightarrow & \text{H—C—C}_2\text{H}_5 \longrightarrow & \text{H—C—C}_2\text{H}_5 \\
| & | & | & | \\
\text{CH}_3 & \text{CH}_3 & \text{CH}_3 & \text{CH}_3
\end{array}
$$

Dextro + 10.35   Dextro + 9.08   Dextro + 19.23   Dextro + 9.5

$$
\begin{array}{ccc}
\text{CH}_2\text{CH}_2\text{COOH} & \text{CH}_2\text{CH}_2\text{CH}_2\text{OH} & \text{CH}_2\text{CH}_2\text{CH}_2\text{Br} \\
| & | & | \\
\text{H—C—C}_2\text{H}_5 \longrightarrow & \text{H—C—C}_2\text{H}_5 \longrightarrow & \text{H—C—C}_2\text{H}_5 \text{————} \\
| & | & | \\
\text{CH}_3 & \text{CH}_3 & \text{CH}_3
\end{array}
$$

Dextro + 13.53   Dextro + 13.70   Dextro + 25.70
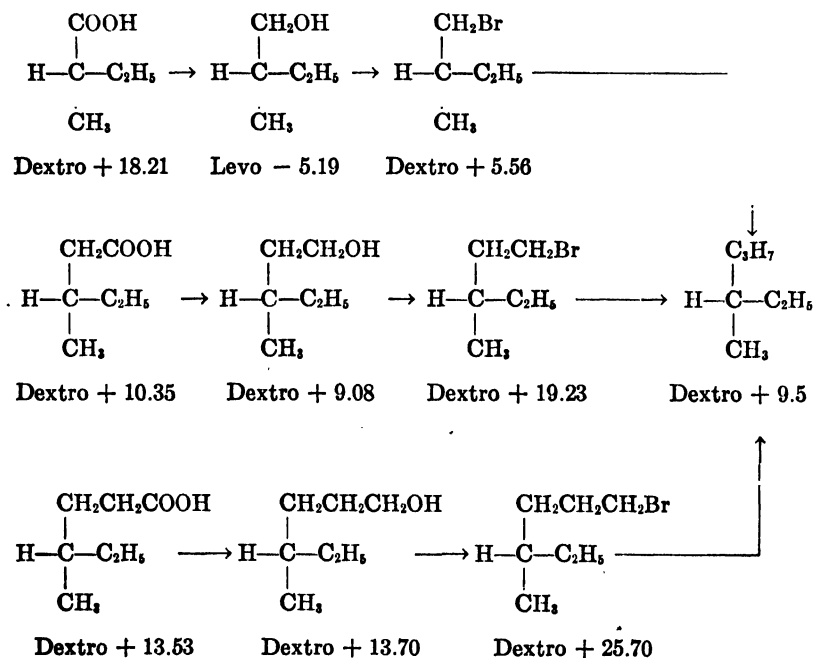
[4] Marckwald, W., *Ber chem. Ges.*, **37**, 1046 (1904).
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[6] Walden, P., *Z. physik. Chem.*, **15**, 638 (1894).

In all of these the allocation of the ethyl group is arbitrary.

The significant features of this series are the following.

1. In the carbinols the direction of rotation is to the left in the first member and to the right in the other. Whether this change is due principally to the increase in mass, or to the distance of the polar group, or to both, cannot as yet be stated.

2. In the acids and halides with a strong polar group, the effect of the polarity seems to overweigh the other factors and hence the direction of rotation remains unchanged from member to member.

3. On passing from the carbinols to the bromides, the direction of change of rotation is the same in all members, namely to the right, and furthermore, the numerical value of the change in every instance is of the same order of magnitude. Thus the change of rotation from carbinol to halide may serve to recognize the configuration of the members of this series.

The question now arises as to the configurations of the 2-substituted butyric acids (4) in which the substituting group is a higher homologue of the radical ethyl. In the series of dextro-2-substituted propionic acids (3), (2,2-disubstituted acetic) series, the changes of rotations from carbinols to halides were always to the right irrespective of the size of the substituting alkyl. On the other hand, in the series described in this paper, namely in the 2-substituted butyric acids (4), the dextro acids in which the substituting alkyl is propyl or a higher homologue lead to a dextro-carbinol and a levo-halide. It was shown above that these acids have a configuration enantiomorphous to dextro-2-ethylbutyric acid (4). Does this then mean that the dextro-2-substituted propionic acids (3) in which the substituting group is a propyl or a higher homologue are enantiomorphously related to the corresponding dextro-2-substituted butyric acids (4)? This question is still in need of an experimental answer.

<div style="text-align:center">EXPERIMENTAL</div>

Dextro-2-Ethylbutyric Acid (4) (Dextro-3-Methylethylpropionic Acid)—The inactive acid was prepared from 2-bromobutane and ethyl malonate.

580 gm. of inactive acid were added to 3 liters of acetone and this heated to boiling on a steam-heated water bath. 1875 gm. of quinine were added and the solution filtered while hot. It was

cooled in ice and crystallization hastened by stirring.   The quinine salt was filtered immediately; otherwise, on standing overnight both forms would crystallize.   The resolution proceeded very slowly and after 10 crystallizations, the quinine salt was decomposed with an excess of 10 per cent hydrochloric acid and the organic acid extracted with ether.   The ether solution was dried with sodium sulfate and the acid distilled.   80 gm. of product were obtained which gave a rotation of   .

$$[\alpha]_D^{25} = \frac{+3.35°}{1 \times 0.923} = +3.63°. \quad [M]_D^{25} = +4.21° \text{ (homogeneous)}$$

700 gm. of inactive 3-methylethylpropionic acid were added to 4 liters of 35 per cent alcohol.   This was warmed on a steam bath and 2750 gm. of brucine were added.   The solution was filtered hot, and the filtrate cooled in an ice-salt mixture, with stirring, until crystallization set in.   It was necessary to filter the brucine salt at about 10° as the salt is very soluble at room temperature.   After three crystallizations the brucine salt was decomposed with 10 per cent hydrochloric acid and the organic acid recovered as described above.   B.p. 105° at 30 mm.; yield 135 gm.; $n_D^{25} = 1.4152$; $D\frac{25}{4} = 0.923$; $\alpha_D^{29} = -4.67°$.

The filtrate from the first crystallization of the brucine salt was evaporated to a heavy syrup, then decomposed with 10 per cent hydrochloric acid.   This gave 102 gm. of acid.

$$[\alpha]_D^{25} = \frac{+3.70°}{1 \times 0.923} = +4.01° \text{ (homogeneous)}$$

3.165 mg. substance: 7.245 mg. CO_2 and 2.920 mg. H_2O.
$C_6H_{12}O_2$.   Calculated.   C 62.02, H 10.42
Found.   " 62.4,   " 10.3

Dextro-Ethyl Ester of 2-Ethylbutyric Acid (4) (*Dextro-Ethyl Ester of 3-Methylethylpropionic Acid*)—160 cc. of absolute alcohol were added to 80 gm. of 2-ethylbutyric acid (4), $[\alpha]_D^{26} = +3.63°$.   To this solution were added 6 cc. of concentrated sulfuric acid and the mixture refluxed 1½ hours on a steam bath.   Some of the excess alcohol was distilled off under reduced pressure and the ester was

extracted from the residue with ether. The ether solution was washed with dilute sodium carbonate solution, then dried with sodium sulfate, and distilled. B.p. 68° at 25 mm.; yield 77 gm.; $D\frac{25}{4} = 0.864; n_D^{25} = 1.4062.$

$$[\alpha]_D^{25} = \frac{+2.82°}{1 \times 0.864} = +3.26°. \quad [M]_D^{25} = +4.69° \text{ (homogeneous)}$$

4.615 mg. substance: 11.295 mg. CO_2 and 4.735 mg. H_2O.
$C_8H_{16}O_2$. Calculated. C 66.55, H 11.17
Found. " 66.7 , " 11.4

Dextro-3-Ethylbutanol (1) (Dextro-3-Methyl-1-Pentanol)—35 gm. of the ethyl ester of 2-ethylbutyric acid (4), $[\alpha]_D^{25} = +3.26°$, were dissolved in 350 cc. of dry ethyl alcohol. This solution was slowly dropped into a suspension of 180 gm. of finely divided sodium in 300 cc. of boiling benzene. This mixture was stirred mechanically and heated while the alcoholic solution of the ester was allowed to flow in at such a rate as to keep the benzene boiling. At the end of the operation, enough alcohol was added to dissolve the excess of sodium. Water was added, and the carbinol extracted with ether. The ether solution was dried over sodium sulfate and then distilled.

The carbinol was purified through its phthalic ester. An equivalent weight of phthalic anhydride was added to the carbinol in 30 cc. of pyridine and the mixture allowed to stand overnight. It was heated an hour on a steam bath, cooled, and acidified with hydrochloric acid. The phthalic ester was extracted with chloroform and the solution dried with anhydrous sodium sulfate. After evaporation of the chloroform under reduced pressure, the residue was dissolved in an excess of sodium carbonate solution. The solution was extracted several times with ether, then acidified with hydrochloric acid, and the phthalic ester extracted with chloroform. This solution was dried with anhydrous sodium sulfate and the chloroform evaporated under reduced pressure. The carbinol was obtained on saponification of the residue with 10 per cent sodium hydroxide. B.p. 72° at 25 mm.; yield 15 gm.; $D\frac{27}{4} = 0.822; n_D^{25} = 1.4182$

$$[\alpha]_D^{27} = \frac{+ \, 2.98°}{1 \times 0.822} = + \, 3.62°. \quad [M]_D^{27} = + \, 3.69° \text{ (homogeneous)}$$

4.000 mg. substance: 10.350 mg. CO_2 and 5.060 mg. H_2O.
$C_6H_{14}O$.   Calculated.   C 70.52, H 13.81
Found.   " 70.56, " 13.87

Dextro-1-Chloro-2-Ethyl Butane (Dextro-1-Chloro-3-Methyl Pentane)—10 gm. of 3-methyl-1-pentanol, $[\alpha]_D^{27} = +3.62°$, were cooled in ice and 30 gm. of thionyl chloride slowly added. The solution was refluxed 1 hour on a steam bath, cooled, and poured on to ice. The chloride was extracted with ether, dried with anhydrous sodium sulfate, and distilled.   B.p. 73° at 100 mm.; $D \dfrac{27}{4} = 0.892$; $n_D^{25} = 1.4210$.

$$[\alpha]_D^{27} = \frac{+ \, 6.65°}{1 \times 0.892} = + \, 7.46°. \quad [M]_D^{27} = + \, 8.95° \text{ (homogeneous)}$$

0.2021 gm. substance: 0.2340 gm. silver chloride.
$C_6H_{13}Cl$.   Calculated. Cl 29.43.   Found.   Cl 28.64

Dextro-2-Ethylbutyric Chloride (4) (Dextro-3-Methylethylpropionyl Chloride)—60 gm. of 2-ethylbutyric acid (4), $[\alpha]_D^{27} = +3.63°$, were cooled in ice and 120 gm. of thionyl chloride added. The mixture was refluxed 1 hour on a steam bath and then fractionated. B.p. 81° at 100 mm.; yield 53 gm.; $D \dfrac{29}{4} = 0.957$; $n_D^{25} = 1.4245$.

$$[\alpha]_D^{29} = \frac{+ \, 5.20°}{1 \times 0.957} = + \, 5.43°. \quad [M]_D^{29} = + \, 7.33° \text{ (homogeneous)}$$

0.1330 gm. substance required 9.70 cc. 0.1 N silver nitrate.
$C_6H_{11}OCl$.   Calculated. Cl 26.37.   Found.   Cl 25.9

Dextro-2-Ethylbutyric Amide (Dextro-3-Methylethyl Propionic Amide)—300 cc. of aqueous ammonia were cooled in ice and 60 gm. of 2-ethylbutyric chloride, $[\alpha]_D^{29} = +5.43°$, were allowed to flow in slowly from a dropping funnel. The solution was stirred during the operation. The amide was filtered, then recrystallized from water. A second crop of crystals was obtained on evaporation of the filtrate to a small volume. The amide was dried overnight under reduced pressure at 65°. Yield 4.5 gm.

$$[\alpha]_D^{26} = \frac{+ \ 0.24° \times 100}{1 \times 20 \times 0.600} = + \ 2.00°. \quad [M]_D^{26} = + \ 2.32° \ \text{(in 75 per cent alcohol)}$$

Dextro-2-Ethylbutyric Nitrile (Dextro-3-Methylethylpropionitrile)
—40 gm. of 2-ethylbutyric amide, $[\alpha]_D^{25} = +2.00°$ (in 75 per cent alcohol), were mixed with 50 gm. of phosphorus pentoxide in a distilling flask. The mixture was heated in a metal bath at 150° under a pressure of 100 mm. until the nitrile discontinued distilling. The product was redistilled. B.p. 87° at 100 mm.; yield 25 gm.;
$D\dfrac{25}{4} = 0.811; n_D^{25} = 1.4070.$

$$[\alpha]_D^{25} = \frac{+ \ 3.02°}{1 \times 0.811} = + \ 3.72°. \quad [M]_D^{25} = + \ 3.41° \ \text{(homogeneous)}$$

4.200 mg. substance: 0.500 cc. N_2 at 26° and 753 mm. •
$C_6H_{11}N$. Calculated. N 14.4. Found. N 13.5

*Dextro-1-Amino-3-Ethyl Butane (Dextro-1-Amino-3-Methyl Pen-
tane)*—22 gm. of 2-ethylbutyric nitrile, $[\alpha]_D^{25} = +3.72°$, were dissolved in 1 liter of absolute alcohol. 90 gm. of metallic sodium were added to this solution in small quantities with stirring. When the sodium had dissolved, the product was cooled and acidified with hydrochloric acid (concentrated). The sodium chloride was filtered, washed with hot alcohol, and the combined filtrates evaporated to dryness under reduced pressure. The residue was dissolved in a minimum amount of water and the amine liberated by adding solid potassium hydroxide. The amine was extracted with ether and the ether solution dried with powdered potassium hydroxide. It was then distilled over sodium. B.p. 67° at 100 mm.; yield 20 gm.; $D\dfrac{26}{4} = 0.767; n_D^{25} = 1.4196.$

$$[\alpha]_D^{26} = \frac{+ \ 3.27° \ ·}{1 \times 0.767} = + \ 4.27°. \quad [M]_D^{26} = + \ 4.31° \ \text{(homogeneous)}$$

4.635 mg. substance: 12.080 mg. CO_2 and 5.995 mg. H_2O.
$C_6H_{15}N$. Calculated. C 71.22, H 14.93
Found. " 71.07, " 14.47

*Levo-2-n-Propylbutyric Acid (4) (Levo-3-Methylpropylpropionic
Acid)*—The inactive acid was prepared from 2-bromopentane and

ethyl malonate. 224 gm. of the inactive acid were dissolved in 1300 cc. of 95 per cent alcohol and heated to boiling on a steam bath. 511 gm. of cinchonidine were added to the hot solution. It was then filtered and 550 cc. of water were added to the filtrate. The salt crystallized by cooling the alcoholic solution in ice. After six crystallizations from 60 per cent alcohol the rotation of the free acid seemed to remain constant. The cinchonidine salt was decomposed with 10 per cent hydrochloric acid and the organic acid extracted with ether. The ether solution was dried with anhydrous sodium sulfate and the acid distilled. B.p. 113° at 17 mm.;

$$D\frac{27}{4} = 0.911; \ n_D^{25} = 1.4214.$$

$$[\alpha]_D^{27} = \frac{-2.30°}{1 \times 0.911} = -2.52°. \quad [M]_D^{27} = -3.28° \text{ (homogeneous)}$$

$$[\alpha]_D^{28} = \frac{-0.37° \times 100}{1 \times 20 \times 0.5442} = -3.4° \text{ (in chloroform)}$$

$$[\alpha]_D^{29} = \frac{-0.61° \times 100}{1 \times 20 \times 0.9752} = -3.1° \text{ (in benzene)}$$

2.875 mg. substance: 6.805 mg. CO_2 and 2.820 mg. H_2O.
$C_7H_{14}O_2$.   Calculated.   C 64.62, H 10.84
            Found.   " 64.54, " 10.97

Levo-Ethyl Ester of 2-n-Propylbutyric Acid (4) (*Levo-Ethyl Ester of 3-Methylpropylpropionic Acid*)—100 cc. of absolute alcohol were added to 40 gm. of 2-*n*-propylbutyric acid (4), $[\alpha]_D^{27} = -2.52°$. To this solution were added 4 cc. of concentrated sulfuric acid. The ester was obtained as described for ethyl ester of 2-ethylbutyric acid (4). B.p. 60° at 10 mm.; yield 43 gm.; $D\frac{27}{4} = 0.806; \ n_D^{30} = 1.4102.$

$$[\alpha]_D^{27} = \frac{-0.34°}{1 \times 0.806} = -0.42°. \quad [M]_D^{27} = -0.66° \text{ (homogeneous)}$$

$$[\alpha]_D^{29} = \frac{-0.30° \times 100}{1 \times 20 \times 0.8076} = -1.86° \text{ (in benzene)}$$

$$[\alpha]_D^{29} = \frac{-0.38° \times 100}{1 \times 20 \times 0.9934} = -1.91° \text{ (in chloroform)}$$

6.070 mg. substance: 15.115 mg. CO_2 and 6.260 mg. H_2O.
$C_9H_{18}O_2$. Calculated. C 68.31, H 11.47
Found. " 67.90, " 11.54

80 gm. of 2-*n*-propylbutyric acid (4), $[\alpha]_D^{26} = -2.19°$, esterified as above gave 85 gm. of ester.

$$[\alpha]_D^{26} = \frac{-0.30°}{1 \times 0.862} = -0.35°. \quad [M]_D^{26} = -0.55° \text{ (homogeneous)}$$

Levo-3-Propyl-1-Butanol (Levo-3-Methyl-1-Hexanol)—28 gm. of ethyl ester of 2-*n*-propylbutyric acid (4), $[\alpha]_D^{27} = -0.42°$, were mixed with 400 cc. of dry alcohol. This was then dropped into a suspension of 46 gm. of sodium in 400 cc. of benzene. The reduction was carried out as described for 3-methyl-1-pentanol. B.p. 80° at 25 mm.; yield 13 gm.; D $\frac{29}{4}$ = 0.8208; n_D^{30} = 1.4202.

$$[\alpha]_D^{29} = \frac{-1.35°}{1 \times 0.8208} = -1.65°. \quad [M]_D^{29} = -1.91° \text{ (homogeneous)}$$

$$[\alpha]_D^{27} = \frac{-0.20° \times 100}{1 \times 20 \times 0.7624} = -1.3° \text{ (in benzene)}$$

$$[\alpha]_D^{27} = \frac{-0.49° \times 100}{2 \times 20 \times 0.7616} = -1.6° \text{ (in chloroform)}$$

3.315 mg. substance: 8.755 mg. CO_2 and 4.125 mg. H_2O.
$C_7H_{16}O$. Calculated. C 72.35, H 13.86
Found. " 72.02, " 13.92

A second portion of ester, $[\alpha]_D^{25} = -0.35°$, was reduced as above. This gave a rotation of

$$[\alpha]_D^{21} = \frac{-1.16°}{1 \times 0.826} = -1.40°. \quad [M]_D^{21} = -1.62° \text{ (homogeneous)}$$

α-Naphthylurethane of Levo-3-Methyl-1-Hexanol—To 1 gm. of α-naphthylisocyanate was added 0.6 gm. of 3-methyl-1-hexanol, $[\alpha]_D^{29} = -1.65°$. The mixture was heated on a steam bath for 10 minutes, then allowed to stand overnight. The urethane was

extracted with hot absolute alcohol, filtered, and placed in the cold room until crystallization.   M.p. 73°.

3.990 mg. substance: 11.050 mg. CO_2 and 2.760 mg. H_2O.
$C_{18}H_{23}NO_2$.   Calculated.   C 75.74, H 8.12
Found.   " 75.52, " 7.74

Dextro-1-Chloro-3-Methyl Hexane—12 gm. of 3-methyl-1-hexanol $[\alpha]_D^{29} = -1.65°$, were treated with 50 gm. of thionyl chloride as described for 1-chloro-3-methyl pentane.   B.p. 66° at 25 mm.;
$D\dfrac{29}{4} = 0.854; n_D^{30} = 1.4282$.

$$[\alpha]_D^{29} = \frac{+3.14°}{1 \times 0.854} = +3.68°. \quad [M]_D^{29} = +4.93° \text{ (homogeneous)}$$

$$[\alpha]_D^{29} = \frac{+0.42° \times 100}{1 \times 20 \times 0.6008} = +3.5° \text{ (in chloroform)}$$

$$[\alpha]_D^{29} \quad \frac{+0.94° \times 100}{1 \times 20 \times 0.8122} \quad +5.78° \text{ (in benzene)}$$

4.455 mg. substance: 10.295 mg. CO_2 and 4.515 mg. H_2O.
0.1360 gm.   "   : 0.1438 gm. AgCl.
$C_7H_{15}Cl$.   Calculated.   C 62.41, H 11.22, Cl 26.37
Found.   " 63.0 , " 11.34, " 26.15

Dextro-1-Bromo-3-n-Propyl   Butane   (Dextro-1-Bromo-3-Methyl Hexane)—30 gm. of 3-*n*-propyl-1-butanol, $[\alpha]_D^{21} = -1.40°$, were cooled in ice and 60 gm. of phosphorus tribromide slowly added with stirring.   The mixture was refluxed 1 hour and then poured on ice.   The halide was extracted with ether.   The ether was evaporated and the residue shaken with cold concentrated sulfuric acid, separated, and washed with water, then dilute sodium carbonate solution.   It was extracted with ether.   The ether solution was dried with anhydrous sodium sulfate and the halide distilled.
B.p. 65° at 20 mm.; yield 39 gm.; $D\dfrac{20}{4} = 1.141$.

$$[\alpha]_D^{21} = \frac{+10.72°}{1 \times 1.141} = +9.39°. \quad [M]_D^{21} = +16.81° \text{ (homogeneous)}$$

3.270 mg. substance: 5.675 mg. CO_2 and 3.455 mg. H_2O.
$C_7H_{15}Br$.   Calculated.   C 46.90, H 7.99
Found.   " 47.32, " 8.40

3-n-Propyl Butane—35 gm. of 1-bromo-3-*n*-propyl butane, $[\alpha]_D^{21}$ = +9.39°, were slowly added to a suspension of 4.5 gm. of finely divided magnesium in 50 cc. of anhydrous ether. The Grignard reagent was poured on ice, and the magnesium hydroxide dissolved in hydrochloric acid. The hydrocarbon was extracted with ether. The ether was distilled and the residue shaken with cold concentrated sulfuric acid. It was separated, washed with water, and extracted from the water solution with a small amount of ether. The ether solution was dried with anhydrous sodium sulfate. The ether was then removed and the residue was fractionated. B.p. 92°; yield 8 gm.; $D\dfrac{21}{4}$ = 0.687; n_D^{25} = 1.3854.

$$[\alpha]_D^{21} = \frac{-5.32°}{1 \times 0.687} = -7.75°. \quad [M]_D^{21} = -7.75° \text{ (homogeneous)}$$

3.080 mg. substance: 9460 mg. CO_2 and 1.430 mg. H_2O.
C_7H_{16}. Calculated. C 83.90, H 16.10
Found. " 83.75, " 16.09

Dextro-2-n-Propylbutyric Chloride (4) (Dextro-3-Methyl-n-Propylpropionyl Chloride)—80 gm. of 2-*n*-propylbutyric acid (4) $[\alpha]_D^{26}$ = − 2.19°, were treated with 150 gm. of thionyl chloride as described for 2-ethylbutyric chloride. B.p. 82° at 50 mm.; yield 87 gm.; $D\dfrac{26}{4}$ = 0.954; n_D^{25} = 1.4293.

$$[\alpha]_D^{26} = \frac{+2.36°}{1 \times 0.954} = +2.47°. \quad [M]_D^{26} = +3.65° \text{ (homogeneous)}$$

0.1300 gm. substance required 8.40 cc. 0.1 N $AgNO_3$.
$C_7H_{13}OCl$. Calculated. Cl 23.90. Found. Cl 22.94

Levo-2-n-Propylbutyric Amide (4) (Levo-3-Methylpropyl Propionic Amide)—80 gm. of 2-*n*-propylbutyric chloride (4), $[\alpha]_D^{26}$ = +2.47°, were converted into the amide by dropping into aqueous ammonia as described for 2-ethylbutyric amide (4). Yield 55 gm.

$$[\alpha]_D^{26} = \frac{-0.25° \times 100}{1 \times 20 \times 0.300} = -4.16°. \quad [M]_D^{26} = -5.36° \text{ (in 75 per cent alcohol)}$$

Dextro-2-n-Propylbutyric Nitrile (4) (Dextro-3-Methyl-n-Propylpropionitrile)—55 gm. of 2-*n*-propylbutyric amide (4), $[\alpha]_D^{26}$ =

−5.36° (in 75 per cent alcohol), were mixed with 75 gm. of phosphorus pentoxide in a distilling flask.   The mixture was heated in a metal bath at 170° under a pressure of 70 mm. until no more nitrile distilled.   The nitrile was then redistilled.   B.p. 95° at 70 mm.; yield 42 gm.; D $\frac{26}{4}$ = 0.810; n_D^{25} = 1.4137.

$$[\alpha]_D^{26} = \frac{+\ 2.66°}{1 \times 0.810} = +\ 3.28°. \quad [M]_D^{26} = +\ 3.64° \text{ (homogeneous)}$$

3.720 mg. substance: 0.397 cc. N_2 at 23° and 755 mm.
$C_7H_{13}N$.   Calculated.   N 12.60.   Found.   N 12.23

Levo-1-Amino-3-Methyl Hexane—35 gm. of 3-methylpropyl-propionitrile, $[\alpha]_D^{26}$ = +3.28°, were reduced by sodium in absolute alcohol and the amine isolated as described for 1-amino-3-methyl pentane.   B.p. 67° at 45 mm.; yield 26 gm.; D $\frac{26}{4}$ = 0.772; n_D^{25} = 1.4249.

$$[\alpha]_D^{26} = \frac{-\ 0.190°}{1 \times 0.772} = -\ 0.25°. \quad [M]_D^{26} = -\ 0.29° \text{ (homogeneous)}$$

0.0906 gm. substance required 7.75 cc. 0.1 N HCl.
$C_7H_{17}N$.   Calculated.   N 12.16.   Found. N 11.97

Levo-2-n-Butylbutyric Acid (4) (Levo-3-Methyl-n-Butylpropionic Acid)—The inactive acid was prepared from 2-bromohexane and ethyl malonate.

720 gm. of the inactive acid were dissolved in 5 liters of hot acetone.   To this boiling solution 1900 gm. of quinine were added. The solution was filtered and the quinine salt of the acid crystallized by cooling in ice and salt.   It was filtered in a cold room.   After seven crystallizations from acetone, the quinine salt was decomposed by shaking with 10 per cent hydrochloric acid.   The organic acid was extracted with ether, dried with sodium sulfate, and then distilled.   This was not resolved to its maximum rotation.   B.p. 131° at 19 mm.; yield 140 gm.; D $\frac{23}{4}$ = 0.909; n_D^{25} = 1.4259.

$$[\alpha]_D^{23} = \frac{-\ 3.45°}{1 \times 0.909} = -\ 3.79°. \quad [M]_D^{23} = -\ 5.45° \text{ (homogeneous)}$$

5.375 mg. substance: 13.225 mg. CO_2 and 5.235 mg. H_2O
$C_8H_{16}O_2$.   Calculated.   C 66.62, H 11.17
Found.   " 67.09, " 10.89

Levo-Ethyl Ester of 2-n-Butylbutyric Acid (4) (*Levo-Ethyl Ester of 3-Methyl-n-Butylpropionic Acid*)—50 gm. of 2-*n*-butylbutyric acid (4), $[\alpha]_D^{23} = -3.79°$, were mixed with 120 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid.   Esterification was carried out as described for ethyl ester of 2-ethylbutyric acid (4).

B.p. 104° at 35 mm.; yield 54 gm.; $D\dfrac{24}{4} = 0.862$, $n_D^{25} = 1.4162$.

$$[\alpha]_D^{24} = \frac{-1.33°}{1 \times 0.862} = -1.54°.\quad [M]_D^{24} = -2.61° \text{ (homogeneous)}$$

3.765 mg. substance: 9.665 mg. CO_2 and 4.000 mg. H_2O.
$C_{10}H_{20}O_2$.   Calculated.   C 69.71, H 11.63
Found.   " 70.03, " 11.89

Levo-3-Methyl-1-Heptanol—50 gm. of ethyl ester of 2-*n*-butylbutyric acid (4), $[\alpha]_D^{24} = -1.54°$, were added to 250 cc. of dry alcohol.   This was dropped into a suspension of 80 gm. of sodium in 375 cc. of boiling toluene with rapid stirring.   The carbinol was isolated from this as described for 3-methyl-1-pentanol.   B.p. 99° at 25 mm.; yield 31 gm.; $D\dfrac{24}{4} = 0.824$; $n_D^{25} = 1.4295$.

$$[\alpha]_D^{24} = \frac{-2.27°}{1 \times 0.824} = -2.75°.\quad [M]_D^{24} = -3.58° \text{ (homogeneous)}$$

4.190 mg. substance: 11.275 mg. CO_2 and 5.155 mg. H_2O.
$C_8H_{18}O$.   Calculated.   C 73.78, H 13.95
Found.   " 73.37, " 13.76

This carbinol was purified through its half phthalic ester but the rotation did not change.

Dextro-1-Bromo-3-n-Butyl   Butane   (*Dextro-1-Bromo-3-Methyl Heptane*)—20 gm. of 3-*n*-butyl-1-butanol, $[\alpha]_D^{24} = -2.75°$, were cooled in ice and 35 gm. of phosphorus tribromide slowly added. The halide was worked up as described for 1-bromo-3-propyl butane.   B.p. 85° at 21 mm.; yield 24 gm.; $D\dfrac{24}{4} = 1.106$; $n_D^{25} = 1.4512$.

$$[\alpha]_D^{24} = \frac{+\ 8.62°}{1 \times 1.106} = +\ 7.79°. \quad [M]_D^{24} = +\ 15.03° \text{ (homogeneous)}$$

4.705 mg. substance: 8.610 mg. CO_2 and 3.810 mg. H_2O.
0.1226 gm.   "   : 0.1199 gm. AgBr.
$\quad\quad$ $C_8H_{17}Br$.   Calculated.   C 49.72, H 8.86, Br 41.43
$\quad\quad\quad\quad\quad\quad$ Found.   " 49.90, " 9.06, " 41.62

Dextro-2-n-Butylbutyric Chloride (4) (Dextro-3-Methyl-n-Butyl-propionyl Chloride)—40 gm. of 2-*n*- butylbutyric acid (4), $[\alpha]_D^{23} = -3.79°$, were treated with 120 gm. of thionyl chloride as described for 3-methylethylpropionyl chloride. B.p. 88° at 30 mm.; yield 43 gm.; $D\dfrac{24}{4} = 0.944; n_D^{25} = 1.4331$.

$$[\alpha]_D^{24} = \frac{+\ 1.84°}{1 \times 0.944} = +\ 1.95°. \quad [M]_D^{24} = +\ 3.16° \text{ (homogeneous)}$$

3.495 mg. substance: 7.595 mg. CO_2 and 2.905 mg. H_2O.
0.1438 gm.   "   : 0.1274 gm. silver chloride.
$\quad\quad$ $C_8H_{15}OCl$.   Calculated.   C 59.22, H 9.29, Cl 21.83
$\quad\quad\quad\quad\quad\quad$ Found.   " 59.26, " 9.30, " 21.93

Levo-2-n-Butylbutyric Amide (4) (Levo-3-Methyl-n-Butyl Pro-pionic Amide)—40 gm. of 2-*n*-butylbutyric chloride (4), $[\alpha]_D^{24} = +\ 1.95°$, were slowly added to 150 cc. of cold aqueous ammonia. The amide was filtered, recrystallized from water, and dried over-night in a vacuum oven at 70°. Yield 30 gm.

$$[\alpha]_D^{24} = \frac{-\ 0.55° \times 100}{1 \times 20 \times 0.4440} = -\ 6.19°. \quad [M]_p^{24} = -\ 8.85° \text{ (in 75 per cent alcohol)}$$

Dextro-2-n-Butylbutyric Nitrile (4) (Dextro-3-Methyl-n-Butyl-Propionitrile)—30 gm. of 2-*n*-butylbutyric amide (4), $[\alpha]_b^{24} = -\ 6.19°$ (in 75 per cent alcohol), were mixed with 50 gm. of phos-phorus pentoxide in a distilling flask. The flask was heated at 160° in a metal bath at 85 mm. until no more nitrile came over. The nitrile was redistilled. B.p. 120° at 85 mm.; yield 22 gm.; $D\dfrac{23}{4} = 0.811; n_D^{25} = 1.4196$.

$$[\alpha]_D^{23} = \frac{+\ 3.51°}{1 \times 0.811} = +\ 4.33°. \quad [M]_D^{23} = +\ 5.41° \text{ (homogeneous)}$$

3.425 mg. substance: 9.780 mg. CO_2 and 3.780 mg. H_2O.
6.730 "   "   : 0.617 cc. N_2 at 23° and 757 mm.
        $C_8H_{15}N$. Calculated. C 76.74, H 12.08, N 11.18
                 Found.   " 77.40, " 12.34, " 10.53

Levo-1-Amino-n-Butyl Butane (Levo-1-Amino-3-Methyl Heptane)
—20 gm. of 2-*n*-butylbutyric nitrile (4) were reduced by 80 gm. of
sodium in 1 liter of absolute alcohol as described for 1-amino-3-
ethyl butane. B.p. 87° at 47 mm.; yield 17 gm.; D $\dfrac{24}{4}$ = 0.782;
n_D^{25} = 1.4288.

$$[\alpha]_D^{24} = \frac{-1.05°}{1 \times 0.782} = -1.34°. \quad [M]_D^{24} = -1.73° \text{ (homogeneous)}$$

3.905 mg. substance: 10.650 mg. CO_2 and 5.155 mg. H_2O.
0.1090 gm.   "   : required 8.54 cc. 0.1 N HCl.
        $C_8H_{19}N$. Calculated. C 74.33, H 14.81, N 10.84
                 Found.   " 74.37, " 14.77, " 10.71

*Levo-2-n-Amylbutyric Acid (4) (Levo-3-Methyl-n-Amylpropionic
Acid)*—The inactive acid was prepared from 3-bromoheptane and
ethyl malonate.

475 gm. of inactive acid were dissolved in 3 liters of hot acetone.
1125 gm. of quinine were added and the solution filtered while hot.
The quinine salt was crystallized by cooling the solution in ice and
salt.   It was filtered in the cold room as it is very soluble in ace-
tone at room temperature.   After eight recrystallizations from
acetone, the quinine salt was decomposed by shaking with 10 per
cent hydrochloric acid.   The organic acid was extracted with ether
and then distilled.   B.p. 135° at 16 mm.; yield 110 gm.; D $\dfrac{23}{4}$ =
0.899; n_D^{25} = 1.4298.

$$[\alpha]_D^{23} = \frac{-4.11°\cdot}{1 \times 0.899} = -4.57°. \quad [M]_D^{23} = -7.13° \text{ (homogeneous)}$$

3.840 mg. substance: 9.685 mg. CO_2 and 3.990 mg. H_2O.
        $C_9H_{18}O_2$. Calculated. C 68.31, H 11.48
                 Found.   " 68.77, " 11.62

*Levo-Ethyl Ester of 2-n-Amylbutyric Acid (4) (Levo-Ethyl Ester of
3-Methyl-n-Amylpropionic Acid)*—50 gm. of 2-*n*-amylbutyric acid

(4), $[\alpha]_D^{23} = -4.57°$, were mixed with 120 cc. of absolute alcohol and 6 cc. of sulfuric acid. The esterification was carried out as described for the ethyl ester of 2-ethylbutyric acid (4). B.p. 117° at 35 mm.; yield 55 gm.; $D\,\dfrac{23}{4} = 0.860$; $n_D^{25} = 1.4200$.

$$[\alpha]_D^{23} = \frac{-1.74°}{1 \times 0.860} = -2.03°. \quad [M]_D^{23} = -3.74° \text{ (homogeneous)}$$

2.685 mg. substance: 6.955 mg. CO_2 and 2.870 mg. H_2O.
$C_{11}H_{22}O_2$.   Calculated.   C 70.89, H 11.93
                Found.   " 70.63, " 11.96

Levo-3-n-Amyl-1-Butanol (Levo-3-Methyl-1-Octanol)—50 gm. of ethyl ester of 2-*n*-amylbutyric acid (4), $[\alpha]_D^{23} = -2.03°$, were added to 250 cc. of dry alcohol. This was dropped into a suspension of 80 gm. of sodium in 375 cc. of boiling toluene. The reduction was carried out as described for 3-*n*-butyl-1-butanol. B.p. 110° at 25 mm.; yield 41 gm.; $D\,\dfrac{24}{4} = 0.827$; $n_D^{25} = 1.4328$.

$$[\alpha]_D^{24} = \frac{-3.09°}{1 \times 0.827} = -3.74°. \quad [M]_D^{24} = -5.39° \text{ (homogeneous)}$$

4.365 mg. substance: 12.065 mg. CO_2 and 5.545 mg. H_2O.
$C_9H_{20}O$.   Calculated.   C 74.92, H 13.90
              Found.   " 75.37, " 14.21

Dextro-1-Bromo-3-Amyl Butane (Dextro-1-Bromo-3-Methyl Octane)—30 gm. of 3-methyl-1-octanol, $[\alpha]_D^{23} = -2.03°$, were cooled in ice and 50 gm. of phosphorus tribromide were slowly added. The halide was prepared and purified as described for 1-bromo-3-methyl heptane. B.p. 104° at 25 mm.; yield 35 gm.; $D\,\dfrac{24}{4} = 1.085$; $n_D^{25} = 1.4536$.

$$[\alpha]_D^{24} = \frac{+6.65°}{1 \times 1.085} = +6.13°. \quad [M]_D^{24} = +13.02° \text{ (homogeneous)}$$

3.725 mg. substance: 7.105 mg. CO_2 and 2.990 mg. H_2O.
0.1479 gm.   "   : 0.1346 gm. AgBr.
       $C_9H_{19}Br$.   Calculated.   C 52.10, H 9.26, Br 38.63
                 Found.   " 52.01, " 8.98, " 38.73

Dextro-2-n-Amylbutyric Chloride (4) (Dextro-3-Methyl-n-Amyl-propionyl Chloride)—40 gm. of 2-n-amylbutyric acid (4), $[\alpha]_D^{23} = - 4.57°$, were cooled in ice and 100 gm. of thionyl chloride were then added. The product was refluxed 1 hour, then fractionated. B.p. 95° at 20 mm.; yield 45 gm.; $D \dfrac{24}{4} = 0.935$; $n_D^{25} = 1.4362$.

$$[\alpha]_D^{24} = \frac{+ 1.27°}{1 \times 0.935} = + 1.36°. \quad [M]_D^{24} = + 2.40° \text{ (homogeneous)}$$

3.045 mg. substance: 6.845 mg. CO_2 and 2.600 mg. H_2O.

$C_9H_{17}OCl$. Calculated. C 61.15, H 9.70, Cl 20.10

Found. " 61.28, " 9.99, " 19.93

Levo-2-n-Amylbutyric Amide (4) (Levo-3-Methyl-n-Amyl Propionic Amide)—The amide was prepared by dropping 40 gm. of 2-n-amylbutyric chloride (4), $[\alpha]_D^{24} = + 1.36°$, into 150 cc. of cold aqueous ammonia. Yield 31 gm.

$$[\alpha]_D^{24} = \frac{- 0.65° \times 100}{0.4858 \times 1 \times 20} = - 6.68°. \quad [M]_D^{24} = - 10.49° \text{ (in 75 per cent alcohol)}$$

Dextro-2-n-Amylbutyric Nitrile (4) (Dextro-3-Methyl-n-Amyl-propionitrile)—30 gm. of 2-n-amylbutyric amide (4), $[\alpha]_D^{24} = - 6.68°$ (in 75 per cent alcohol), were mixed with 50 gm. of phosphorus pentoxide. The mixture was heated in a distilling flask at 160° and 85 mm. pressure until the nitrile ceased distilling. It was redistilled. B.p. 135° at 85 mm.; yield 21 gm.; $D \dfrac{23}{4} = 0.813$; $n_D^{25} = 1.4239$.

$$[\alpha]_D^{23} = \frac{+ 3.27°}{1 \times 0.813} = + 4.02°. \quad [M]_D^{23} = + 5.59° \text{ (homogeneous)}$$

3.265 mg. substance: 9.265 mg. CO_2 and 3.495 mg. H_2O.

0.4360 gm. " : 4.10 cc. N_2 at 25° and 757 mm.

$C_9H_{17}N$. Calculated. C 77.64, H 12.30, N 10.07

Found. " 77.39, " 11.97, " 10.36

Levo-1-Amino-3-Amyl Butane (Levo-1-Amino-3-Methyl Octane)—20 gm. of 2-n-amylbutyric nitrile (4) were reduced by 80 gm. of

sodium in 1 liter of absolute alcohol as described for 1-amino-3-

methyl pentane.   B.p. 87° at 19 mm.; yield 16 gm.; D $\dfrac{24}{4}$ = 0.788;

n_D^{25} = 1.4326.

$$[\alpha]_D^{24} = \frac{-1.75°}{1 \times 0.788} = -2.22°. \quad [M]_D^{24} = -3.17° \text{ (homogeneous)}$$

3.760 mg. substance: 10.375 mg. CO_2 and 4.685 mg. H_2O.
$C_9H_{21}N$.   Calculated.   C 75.41, H 14.79
Found.   " 75.24, " 13.94

Dextro-Ethyl Ester of 2-n-Hexylbutyric Acid (Dextro-Ethyl Ester of 3-Methyl-n-Hexylpropionic Acid)—60 gm. of 2-*n*-hexylbutyric acid, $[\alpha]_D^{26}$ = + 0.78°, were mixed with 120 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid.   The esterification was carried out as described for ethyl ester of 2-ethylbutyric acid (4).

B.p. 135° at 36 mm.; yield 65 gm.; D $\dfrac{25}{4}$ = 0.862; n_D^{25} = 1.4232.

$$[\alpha]_D^{25} = \frac{+0.33°}{1 \times 0.862} = +0.38°. \quad [M]_D^{25} = +0.75° \text{ (homogeneous)}$$

3.330 mg. substance: 8.850 mg. CO_2 and 3.655 mg. H_2O.
$C_{12}H_{24}O_2$.   Calculated.   C 71.93, H 12.09
Found.   " 72.47, " 12.28

Dextro-3-Methyl-1-Nonanol—50 gm. of ethyl ester of 2-*n*-hexyl-butyric acid (4), $[\alpha]_D^{25}$ = + 0.38°, were reduced by dissolving in 300 cc. of absolute alcohol and dropping this solution into a suspension of 80 gm. of sodium in boiling toluene.   B.p. 122° at 24

mm.; yield 35 gm.; D $\dfrac{23}{4}$ = 0.837; n_D^{25} = 1.4348.

$$[\alpha]_D^{23} = \frac{+0.36°}{1 \times 0.837} = +0.43°. \quad [M]_D^{23} = +0.67° \text{ (homogeneous)}$$

3.185 mg. substance: 8.810 mg. CO_2 and 3.945 mg. H_2O.
$C_{10}H_{22}O$.   Calculated.   C 75.85, H 14.03
Found.   " 75.43, " 13.86

Dextro-2-n-Hexylbutyric Acid (4) (3-Methyl-n-Hexylpropionic Acid)—The inactive acid was prepared from 2-bromooctane and ethyl malonate.

516 gm. of the inactive acid were dissolved in 2 liters of hot acetone. 1134 gm. of quinine were added and the solution filtered. It was very difficult to get the alkaloid salt to crystallize. It was recrystallized by stirring in ice and salt and filtered at 0°. After eight crystallizations the salt was decomposed and the acid recovered as described for 2-n-amylbutyric acid (4). B.p. 133° at 8 mm.;

$$D \frac{26}{4} = 0.899; n_D^{25} = 1.4339.$$

$$[\alpha]_D^{25} = \frac{+ 0.71°}{1 \times 0.899} = + 0.78°. \quad [M]_D^{25} = + 1.32° \text{ (homogeneous)}$$

2.565 mg. substance: 6.595 mg. CO_2 and 2.700 mg. H_2O.
$C_{10}H_{20}O_2$. Calculated. C 69.70, H 11.70
Found. " 70.11, " 11.78

Levo-1-Bromo-3-n-Hexyl Butane (Levo-1-Bromo-3-Methyl Nonane)—20 gm. of 3-n-hexyl-1-butanol were cooled in ice and 30 gm. of phosphorus tribromide added. The bromination was carried out as described for 1-bromo-3-methyl heptane. B.p. 116° at 21 mm.; yield 19 gm.; $D \frac{25}{4} = 1.063; n_D^{25} = 1.4556.$

$$[\alpha]_D^{25} = \frac{- 0.73°}{1 \times 1.063} = - 0.69°. \quad [M]_D^{25} = - 1.56° \text{ (homogeneous)}$$

5.085 mg. substance: 10.250 mg. CO_2 and 4.480 mg. H_2O.
$C_{10}H_{21}Br$. Calculated. C 54.24, H 9.60
Found. " 54.96, " 9.85

Dextro-1-Bromo-3-n-Ethyl Butane (Dextro-1-Bromo-3-Methyl Pentane)—200 gm. of phosphorus tribromide were added to 180 gm. of cold 3-methyl-1-pentanol, $[\alpha]_D^{27} = + 1.67°$. The bromination was carried out as described for 1-bromo-3-methyl heptane. B.p. 80° at 85 mm.; yield 200 gm.; $D \frac{23}{4} = 1.171; n_D^{25} = 1.4415.$

$$[\alpha]_D^{25} = \frac{+ 3.70°}{1 \times 1.171} = + 3.16°. \quad [M]_D^{25} = + 5.21° \text{ (homogeneous)}$$

4.810 mg. substance: 7.680 mg. CO_2 and 3.255 mg. H_2O.
0.1464 gm. " : 0.1674 gm. silver bromide.
$C_6H_{13}Br$. Calculated. C 43.60, H 7.93, Br 48.45
Found. " 43.54, " 7.57, " 48.68

Dextro-4-Methyl-1-Hexanol—200 gm. of 1-bromo-3-ethyl butane, $[\alpha]_D^{23} = + 3.16°$, were slowly added to 28 gm. of magnesium in 300 cc. of ether. To this was added 60 gm. of paraformaldehyde. The Grignard reagent was poured on to a mixture of ice and hydrochloric acid and the carbinol extracted with ether. It was purified through its half phthalic ester. B.p. 77° at 20 mm.; yield 56 gm.; $D\dfrac{23}{4} = 1.809; n_D^{25} = 1.4233$.

$$[\alpha]_D^{23} = \frac{+ 1.57°}{1 \times 0.809} = + 1.94°. \quad [M]_D^{23} = + 2.25° \text{ (homogeneous)}$$

3.510 mg. substance: 9.270 mg. CO_2 and 4.365 mg. H_2O.
$C_7H_{16}O$.   Calculated.   C 72.5, H 13.8
            Found.   " 72,   " 13.9

Dextro-1-Bromo-4-Ethyl Pentane (Dextro-1-Bromo-4-Methyl Hexane)—This halide was prepared from 57 gm. of 4-methyl-1-hexanol, $[\alpha]_D^{23} = + 1.94°$ and 95 gm. of phosphorus tribromide as described for 1-bromo-3-methyl pentane. B.p. 78° at 44 mm.; yield 58 gm.; $D\dfrac{27}{4} = 1.070$.

$$[\alpha]_D^{27} = \frac{+ 2.52°}{1 \times 1.070} = + 2.36°. \quad [M]_D^{27} = + 4.22° \text{ (homogeneous)}$$

4.850 mg. substance: 8.445 mg. CO_2 and 3.625 mg. H_2O.
0.1482 gm.   "   : 0.1563 gm. silver bromide.
$C_7H_{15}Br$.   Calculated.   C 46.90, H 8.43, Br 44.67
           Found.   " 47.48, " 8.36, " 44.88

Dextro-3-Methyl Hexane—52 gm. of 1-bromo-4-methyl hexane, $[\alpha]_D^{27} = + 2.36°$, were slowly dropped into 7.2 gm. of magnesium in 75 cc. of dry ether. The solution was poured on to a mixture of ice and hydrochloric acid and the hydrocarbon worked up as described for levo-3-methyl hexane. B.p. 92°; yield 21 gm.; $D\dfrac{24}{4} = 0.684; n_D^{25} = 1.3854$.

$$[\alpha]_D^{24} = \frac{+ 1.14°}{1 \times 0.684} = + 1.67°. \quad [M]_D^{24} = + 1.67° \text{ (homogeneous)}$$

3.185 mg. substance: 9.775 mg. CO_2 and 4.510 mg. H_2O.
C_7H_{16}.   Calculated.   C 83.90, H 16.10
                 Found.   " 83.69, " 15.84

Levo-4-Butyl-1-Pentanol (Levo-4-Methyl-1-Octanol)—A Grignard reagent was prepared from 11 gm. of magnesium in ether and 80 gm. of 1-bromo-3-methyl heptane, $[\alpha]_D^{24} = + 7.79°$. To this was added 20 gm. of paraformaldehyde and the carbinol isolated as described for 3-methyl-1-pentanol. B.p. 106° at 17 mm.; yield 33 gm.; D $\dfrac{27.5}{4}$ = 0.820; n_D^{25} = 1.4335.

$$[\alpha]_D^{27.5} = \frac{-0.37°}{1 \times 0.820} = -0.45°. \quad [M]_D^{27.5} = -0.65° \text{ (homogeneous)}$$

2.710 mg. substance: 7.480 mg. CO_2 and 3.375 mg. H_2O.
$C_9H_{20}O$.   Calculated.   C 74.92, H 13.9
                 Found.   " 75.26, " 13.93

Dextro-1-Bromo-4-Octyl Pentane (Dextro-1-Bromo-4-Methyl Octane)—This was prepared from 33 gm. of 4-methyl-1-octanol, $[\alpha]_D^{27.5} = -0.45°$, and 50 gm. of phosphorus tribromide as described for 1-bromo-3-methyl pentane. B.p. 95° at 17 mm.; yield 37 gm.; D $\dfrac{27.5}{4}$ = 1.089; n_D^{25} = 1.4540.

$$[\alpha]_D^{27.5} = \frac{+3.95°}{1 \times 1.089} = +3.63°. \quad [M]_D^{27.5} = +7.51° \text{ (homogeneous)}$$

4.955 mg. substance: 9.500 mg. CO_2 and 4.220 mg. H_2O.
$C_9H_{19}Br$.   Calculated.   C 52.10, H 9.26
                 Found.   " 52.28, " 9.53

Levo-4-Butyl Pentane (Levo-4-Methyl Octane)—17 gm. of 1-bromo-4-methyl octane, $[\alpha]_D^{27.5} = + 3.63°$, were reduced by forming a Grignard reagent and pouring into water as described for 3-methyl hexane. B.p. 141° at 760 mm.; yield 7 gm.; D $\dfrac{19}{4}$ = 0.717.

$$[\alpha]_D^{19} = \frac{-0.76°}{1 \times 0.717} = -1.06°. \quad [M]_D^{19} = -1.36° \text{ (homogeneous)}$$

2.915 mg. substance: 9.000 mg. CO_2 and 4.105 mg. H_2O.
C_9H_{20}.   Calculated.   C 84.28, H 15.72
                 Found.   " 84.19, " 15.75

Levo-4-Amyl-1-Pentanol (Levo-4-Methyl-1-Nonanol)—This car-
binol was prepared by the action of 12 gm. of paraformaldehyde on
a Grignard reagent formed from 60 gm. of 1-bromo-3-methyl
octane, $[\alpha]_D^{24} = +6.13°$, as described for 3-methyl-1-pentanol.
B.p. 120° at 17 mm.; yield 27 gm.; $D \dfrac{27}{4} = 0.826$; $n_D^{25} = 1.4364$.

$$[\alpha]_D^{\pi} = \frac{-1.20°}{1 \times 0.826} = -1.45°. \quad [M]_D^{\pi} = -2.29° \text{ (homogeneous)}$$

3.230 mg. substance: 9.030 mg. CO_2 and 4.045 mg. H_2O.
$C_{10}H_{22}O$.   Calculated.   C 75.85, H 14.03
            Found.   " 76.23, " 14.01

*Dextro-1-Bromo-4-Amyl Pentane (Dextro-1-Bromo-4-Methyl No-
nane)*—20 gm. of 4-methyl-1-nonanol, $[\alpha]_D^{27} = -1.45°$, were cooled
in ice and 30 gm. of phosphorus tribromide added. The halide
was isolated and purified as described for 1-bromo-3-methyl
heptane.   B.p. 115° at 17 mm.; yield 22 gm.; $D \dfrac{27.5}{4} = 1.081$.

$$[\alpha]_D^{\pi.\,\imath} = \frac{+2.72°}{1 \times 1.081} = +2.52°. \quad [M]_D^{\pi.\,\imath} = +5.57° \text{ (homogeneous)}$$

Levo-3-Amyl Butane (Levo-3-Methyl Octane)—A Grignard rea-
gent was formed from 6 gm. of magnesium in ether and 50 gm. of
1-bromo-3-methyl octane, $[\alpha]_D^{24} = +6.23°$.   The Grignard reagent
was divided into two parts.   To the first part was added bromine
in ether.   This was to form the original bromide in order to test
whether racemization took place during the formation of the Grig-
nard reagent.   The halide had a rotation of

$$[\alpha]_D^{\,\mathit{36}} = \frac{+6.71°}{1 \times 1.085} = +\bar{.}6.17° \text{ (homogeneous)}$$

4.415 mg. substance: 8.465 mg. CO_2 and 3.700 mg. H_2O.
$C_9H_{19}Br$.   Calculated.   C 52.10, H 9.26
            Found.   " 52.28, " 9.37

The second portion of the Grignard reagent was poured on to
ice and the hydrocarbon extracted and purified as described for 3-

methyl hexane.   B.p. 143°; yield 19 gm.; D $\dfrac{27}{4}$ = 0.714; n^{25}
1.4052.

$$[\alpha]_D^{27} = \dfrac{-6.10°}{1 \times 0.714} = -8.5° \text{ (homogeneous)}$$

2.525 mg. substance: 7.810 mg. CO_2 and 3.560 mg. H_2O.
C_9H_{20}.   Calculated.   C 84.27, H 15.73
Found.   " 84.34, " 15.77

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THE UNSAPONIFIABLE LIPIDS OF LETTUCE

I. CAROTENE*

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The recent renewal of interest in the relation of vitamin A to the plant pigment carotene recalls the observations of Steenbock through which he and his coworkers were able to relate the yellow pigments of corn (25), peas (27), and other vegetables (26) with vitamin A, while in animal fats, notably in cod liver oil (28), there was no such association. Simultaneous and subsequent reports from abroad (3, 5, 7) in part confirmed these statements but the identification of vitamin A with carotene was discouraged by the observations of Stephenson (29), whose proper explanation is now apparent (21), and especially by the critical review of Palmer (22). The subject was reopened by the evidence offered by von Euler and his coworkers (9) in 1928, to which they have added further proofs (8, 10, 11), and with the recent and convincing experiments of English workers, especially Moore (20), Capper (2), and latterly Drummond (4), and the work of Javillier and Emerique (13, 14), it can no longer be doubted that carotene is the plant source of the vitamin A of animal tissue. The confirmation of this relationship tends to harmonize some discordant facts and presents further interesting problems, especially the chemical reactions of carotene and the mechanism of its transformation to vitamin A. The purpose of this paper is to record a few observations upon the properties of carotene and to present further proof of the vitamin A activity of this substance obtained from another vegetable source, lettuce.

During the course of an investigation of the unsaponifiable lipids of lettuce, some brilliant, red, pleochroic crystals separated from a methyl alcohol solution. These crystals proved to be carotene. Approximately 200 mg. were isolated from 10 kilos of dried lettuce, representing 140 kilos of fresh leaves. This yield is probably only a small fraction of the original content, the rest of which was destroyed in the preparation of the material or removed in previous fractions. The method of preparation of the leaves, their extraction, saponification, and fractionation will be described in a later paper.

Physical and Chemical Properties

The solubility of lettuce carotene in various organic solvents and its color reactions are similar to those reported for carotene obtained from other sources.

The melting point of the first crystals obtained was 173-174° (corrected); after three recrystallizations, 180° (corrected). The small amount of material available prohibited further recrystallization. The two preparations showed equal physiological activity. The melting point of pure carotene was recently reported as 184-185° (6, 14). The most satisfactory method of purification consists in throwing down the carotene from a chloroform solution by the addition of an excess of methyl alcohol. This method is rapid and seemingly efficient.

The crystalline forms of carotene are many and varied, depending primarily on the solvent used but also upon temperature, concentration, rate of cooling, and duration of contact with the solvent. We have obtained rhombohedrons from petroleum ether, triangular plates from acetone, needles from chloroform and methyl alcohol, clusters of needles from carbon disulfide and absolute alcohol (Fig. 1), square plates from petroleum ether and methyl alcohol, and other complex figures. Whatever the external form, carotene crystals are hexagonal.[1] Kohl (17) called them orthorhombic, but the impurities in his carotene (m.p. 168-169°) might account for the disagreement.

Exposure of carotene crystals in the laboratory caused them to lose their color; the bleaching began at the outer edges and pro-

[1] We are indebted to Dr. J. J. Runner of the Geology Department, State University of Iowa, for the crystal analyses.

gressed toward the center, a sharp line of demarcation separating the bleached (or achroocarotene) from the unchanged portion (Fig. 2). The rapidity of decolorization was increased by heating and crystals sufficiently small could be completely transformed in 30 hours at 105°.

Frequent reference is made in the literature to the rapid decolorization of carotene crystals, which has been considered the result of oxidation. Indeed, weight increases of 21 to 37.9 per cent of the original carotene have been reported (1, 17, 30, 31). That the decolorization which we observed (at 105°) is probably not an oxidation is indicated by the following facts: The trans-

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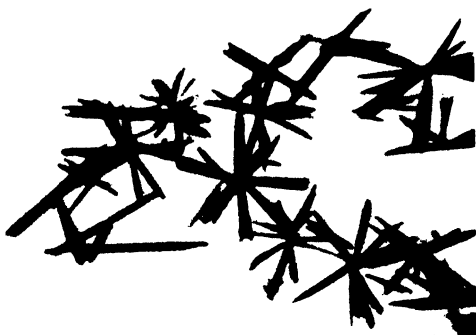
Fig. 1   Fig. 2
</div>

Fig. 1. Carotene crystals from carbon disulfide and absolute alcohol.

Fig. 2. Partially bleached carotene crystals, originally from petroleum ether.

formation was no more rapid in oxygen than in air and as rapid in nitrogen or carbon dioxide or under glycerol, as in oxygen; there was no change in weight.

3.9 mg. of carotene on a small watch crystal were placed in a small desiccator, and evacuated by a vacuum pump to 0.1 mm. of pressure (or less); the desiccator was then filled with nitrogen from a tank, and reevacuated. After the fourth such evacuation nitrogen was admitted to a pressure of approximately 400 mm. The desiccator was placed in an oven maintained at 105°. After 22 hours, it was cooled, opened, and the carotene weighed; weight, 3.9 mg. Decolorization was approximately 70 per cent complete.

A few crystals of carotene were treated similarly, CO_2 being used as the inert gas.   Decolorization was complete in 30 hours.

1.4 mg. of carotene were placed in a desiccator which was filled with oxygen.   After 30 hours, the crystals were weighed; weight, 1.4 mg.   Approximately 70 per cent had been bleached.

The external form of the original crystals was preserved but the crystal system became isometric.   Transformation of crystal system without change in external crystal structure is indicative of unusual molecular mobility.   The complex formula for carotene recently submitted by Karrer and his coworkers (16), and which is in harmony with Smith and Spoehr's observations (24), suggests the possibility of molecular rearrangement, or even polymerization.   The addition of oxygen noted by other workers may be a normal mode of decomposition which, at higher temperatures, is replaced by a rearrangement independent of the gaseous phase in contact; or it may have been caused by accompanying impurities which directed the decomposition of their preparations.

The crystals of achroocarotene differ in solubility from carotene. They are soluble in acetone and methyl alcohol, but relatively insoluble in the natural or synthetic oils.   With concentrated sulfuric acid, carotene gives a deep blue color, achroocarotene a red-brown.   Achroocarotene shrinks at 159°, and melts at 167–168° (uncorrected).   The bleached carotene of previous workers melted at 120–125° (17).   Achroocarotene lacks the odor of violets which is known to accompany the oxidation of carotene (15, 22), and is stable toward oxygen.

1.35 mg. of achroocarotene were exposed to an atmosphere of oxygen at 105° for 60 days.   There was no change in physical properties or in weight.

Achroocarotene has no physiological activity as vitamin A.

The bleaching of carotene in various solvents has proved to be of exceptional interest.   A study of the stability of carotene solutions was suggested by the contention of Drummond (6) that synthetic solvents were preferable to natural oils for administering carotene to animals.   He first proposed ethyl oleate, and using this solvent, he denied the vitamin A efficacy of carotene.   Hume and Maclean (12) found that ethyl oleate solutions are unstable, and Drummond (4) then recognized his error, retracted his denial, and offered ethyl laurate as the ideal solvent.

Carotene solutions of the same strength as those used in the biological assays, that is containing 1 mg. in 200 drops of the solvent, were compared colorimetrically with a solution of potassium dichromate (approximately 14 gm. per liter). They were allowed to stand at room temperature and exposed to the air. The curves for the fading of carotene in ethyl oleate, ethyl laurate, ethyl oleate with hydroquinone, and ethyl laurate with hydroquinone, are given in Chart I. It is at once apparent that ethyl laurate is a solvent of doubtful value. A solution of carotene in ethyl laurate with hydroquinone is very stable; there is
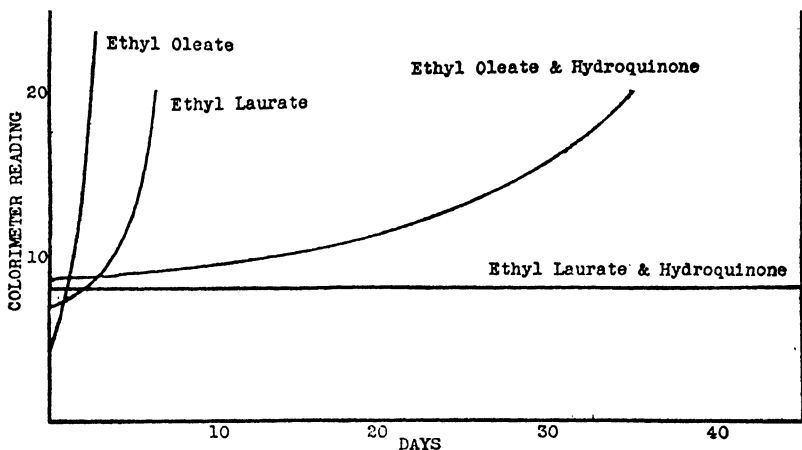
CHART I.   Fading of carotene solutions at room temperature and exposed to the air, measured against standard $K_2Cr_2O_7$. Concentration of carotene, 0.005 mg. per drop; hydroquinone when used, 0.025 mg. per drop.

no fading of color in 45 days at room temperature and exposed to the air. If such a solution is kept stoppered, dark, and in the cold, it remains constant for months. The hydroquinone, present in our solutions in 5 times the concentration of carotene (i.e. 5 mg. in 200 drops) has no effect on the test animals at this level as shown conclusively by unpublished observations. Carotene solutions in cottonseed oil are relatively stable; the rate of fading is comparable to that in ethyl laurate with hydroquinone.

At higher temperatures (105°), bleaching is complete in a few hours in ethyl laurate and in ethyl oleate, but is delayed by the

presence of hydroquinone.   Solutions of carotene in such stable
solvents as mineral oil and xylene bleach similarly, though not
as rapidly, but here again the presence of hydroquinone efficiently
protects the carotene.   If the decolorization of carotene is pre-
vented by an antioxidant, the destructive process would appear
to be an oxidation, yet hot xylene is not an oxidizing medium, for
the vapor effectually prevents air from contact with the solution.
Furthermore the decolorization of a chloroform solution of caro-
tene at room temperature, and in the absence of air, proceeds

TABLE I

Prooxidant Capacity of Carotene and Achroocarotene

Autoxidizable mixture: 5 gm. lard, 10 drops cod liver oil	Induction period	Index*
	hrs.	
	6	
+ 5 mg. carotene......................	3½	0.58
	9	
+ 5 mg. carotene......................	3½	0.40
	3	
+ 5 mg. carotene......................	1½	0.50
	11½	
+ 3 mg. achroocarotene.................	9½	0.83
	6	
+ 4.2 mg. achroocarotene...............	4½	0.75

* The duration of the induction period with test substance (anti- or
prooxidant) divided by the induction period of the autoxidizable fat
alone has been called the index (18).

very rapidly under the influence of ultra-violet light, and is de-
layed by the presence of hydroquinone.   If the bleaching of solu-
tions of carotene parallels the bleaching of the crystals at high
temperatures, the process apparently takes place without the
intervention of oxygen and the inactivation of carotene as vitamin
A, under these conditions, is not the result of oxidation.

    These considerations are perhaps related to the activity of
carotene as a prooxidant.   The active uptake of oxygen by an
autoxidizable substance is preceded by an interval of variable

length known as the induction period. The length of this induction period is a measure of oxidizability. Antioxidants prolong the induction period, prooxidants shorten it. Carotene is an active prooxidant, as shown in Table I.

Of a number of organic compounds thus far examined in this laboratory (18), carotene is the only one that has shown this property. All the others were either antioxidants or inactive. The prooxidant capacity of achroocarotene appears to be less than that of carotene, but the relative insolubility of achroocarotene in the natural oils used for the tests prevents strict comparison.

Physiological Properties

The physiological activity of carotene was determined by the curative method. Rats not standardized, but available from other experiments on diets[2] lacking vitamin A, were used after their growth had ceased, and their weight was rapidly declining. At this point ophthalmia was usually marked, although not always, and other characteristic symptoms were present. Carotene in doses of 0.005 mg. per day was always sufficient, except in rats suffering from other abnormalities, to cause a marked increase in weight, accompanied by recovery from the ophthalmia. Cases which failed to respond to carotene treatment invariably showed other abnormalities; urinary calculi were often present. The rate of recovery varied considerably for the individual rat. Undoubtedly the following factors all play an important part: the age, weight, condition and idiosyncrasy of the animal, the age of the carotene preparation, the conditions under which it has been kept, and the solvent in which it is administered. Perhaps there are others, more obscure. Since so many factors are present in such a study, one hesitates to point to this or that variable as responsible for an unusual reaction to treatment. The best that can be done is to determine the minimum response and the average for a large number of animals under various conditions.

The effect of carotene administration may not be noticeable

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[2] The diets all contained alcohol-extracted casein, sucrose, lard, mineral salts, yeast, and viosterol. For the last two constituents, we are indebted to the Northwestern Yeast Company and to Mead Johnson and Company, respectively. The additional constituents of some of the diets did not prevent the ultimate exhaustion of the vitamin A stores of the animals.

Lipids of Lettuce.   I

TABLE II

Effect of Carotene on Growth of Vitamin A-Deficient Rats

Rat No.	Age	Weight	Carotene solution administered (0.005 mg. per drop)	Duration of carotene feeding	Weight gained	Duration of gain	Weight gained per drop	Carotene solution No.*
	days	*gm.*	*drops*	*days*	*gm.*	*days*	*gm.*	
123	110	101	14	14	103	52	7.3	1
	172	179	10	10	48	31	4.8	1
	224	212	10	6	28	30	2.8	6
124	171	109	28	11	44	17	1.6	1
	195	129	5	5	44	17	8.8	6
125	136	117	10	6	24	15	2.4	2
126	76	65	48	25	115	48	2.4	1
127	123	72	7	7	58	36	8.3	1
131	99	83	23	23	108	34	4.7	1
	164	133	30	15	87	31	2.9	5
	220	169	9	5	31	17	3.4	6
134	76	56	56	27	184	58	3.3	1
	161	200	34	17	56	30	1.6	1
	215	176	4	3	13	4	3.2	6
137	124	118	5	5	69	25	13.8	1
	184	165	12	11	50	16	4.2	6
138	124	106	5	5	69	21	13.8	1
	158	136	18	18	53	20	2.9	4
140	136	121	10	7	85	27	8.5	1
188	60	58	26	26	93	48	3.6	1
	131	119	16	14	47	22	2.9	5
191	88	80	31	16	68	30	2.2	3
193	87	95	6	6	40	18	6.7	5
	127	101	10	2	40	14	4.0	6
194	74	103	12	12	33	17	2.7	4
197	87	90	17	16	55	50	3.2	5
	193	93	5	1	13	2	2.6	7
198	64	70	11	9	50	22	4.5	1
205	200	111	3	3	22	4	7.3	14
	206	121	3	1	19	4	6.3	14
209	207	162	5	5	12	9	2.4	14
210	171	127	9	9	35	14	3.9	14
214	162	93	5	5	60	29·	12.0	14
215	115	125	23	23	53	35	2.3	9
216	121	135	10	2	43	15	4.3	7
217	115	110	12	12	22	21	1.8	9
222	162	125	12	12	53	15	4.4	14
	198	145	5	5	16	8	3.2	14

TABLE II—*Concluded*

Rat No.	Age	Weight	Carotene solution administered (0.005 mg. per drop)	Duration of carotene feeding	Weight gained	Duration of gain	Weight gained per drop	Carotene solution No.[*]
	days	*gm.*	*drops*	*days*	*gm.*	*days*	*gm.*	
224	162	127	12	12	72	25	6.0	14
	198	172	5	5	22	8	4.4	14
225	183	114	4	4	12	6	3.0	14
Total.............			597		2195			
Average...							3.68	

* The following solvents were used: cottonseed oil in Solutions 1, 5, 6, 7; ethyl oleate containing 0.1 per cent hydroquinone in Solutions 3 and 9; ethyl oleate, freshly prepared solution of recrystallized carotene, in Solution 2; ethyl oleate, 0.1 per cent hydroquinone, thrice recrystallized carotene, in Solution 4; ethyl laurate, 0.1 per cent hydroquinone, in Solution 14.

for a day or two, after which growth is resumed. After the cessation of dosing, animals continue to grow for a variable length of time. Large doses, given singly or in two portions, have effects comparable to those obtained with the same amount of carotene given in small doses over a number of days, the average growth being more or less constant for a given amount. Table II lists all of the forty-one cures, some repeated on the same rat; Chart II contains a few typical graphs which visualize the rapidity of the recovery. It is to be especially noted that although conditions differed widely, most of the cases average 3 to 5 gm. gain per 0.005 mg. of carotene (the amount contained in the ordinary 1 drop dose). A quantitative relationship seems to exist between the absolute amount of carotene administered and the growth it permits.

Sherman (23) has defined the unit of vitamin A as the amount sufficient to cause an increment of 3 gm. in weight in 1 week. The time element in our measurements varied widely, the response to a dosage sometimes taking place over a period of 3 to 4 days, at other times over 2 weeks being required for the rat to attain its maximum weight. This factor being neglected, 0.005 mg. of carotene may be said to be approximately equivalent to a unit.

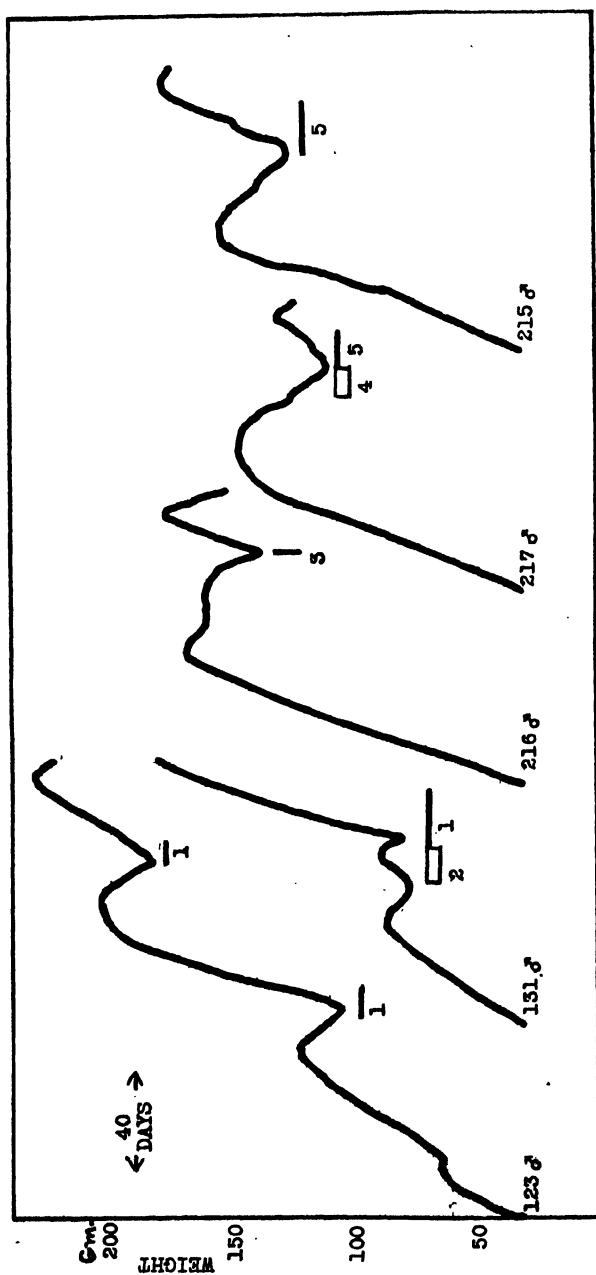CHART II. Effect of carotene on the growth of vitamin A-deficient rats. 0.005 mg. of carotene per drop of solvent. The numbered lines indicate vertically the number of drops administered per day, and horizontally the duration of the dosage. The following solvents were used: Solvents 1 and 3, cottonseed oil (CSO); Solvent 2, inactive material in CSO; Solvent 4, ethyl oleate; Solvent 5, ethyl oleate plus hydroqunone (0.1 per cent).

It would seem possible to maintain a rat at slow growth (especially in rearing experiments) on less than 0.005 mg. per day, but this has been successful only in isolated cases.

DISCUSSION

Since carotene in the solid state is decolorized and rendered inert, physiologically, by heat in the absence of oxygen, and since in solution it undergoes this change even more rapidly under the influence of heat or ultra-violet radiation, it follows that the resultant achroocarotene is not a product of oxidation but rather of an intramolecular rearrangement, or possibly of polymerization. Inasmuch as hydroquinone delays the transformation of carotene under these conditions, its function as an antioxidant must be extended to include the capacity to prevent the shift in electrons, if such it be, which attends the thermal or photoelectric change of the unstable to the colorless and more stable form of carotene.

The prooxidant character of carotene, a capacity found in smaller degree in achroocarotene, demonstrates that the former possesses greater molecular energy than the latter. The electronic significance of conjugate double bonds, of which carotene possesses eleven, is, as yet, obscure. It is also not possible to state whether carotene is itself oxidized when it hastens oxidation of an autoxidizable substance. Since achroocarotene retains some of its prooxidant activity, it is probably not the end-product of the change in the presence of autoxidizable materials, although an antioxidant (anthraquinone (19)) has been recovered unchanged from a reaction which it delayed.

Obviously the commonly measured stability of vitamin A (carotene) in various vegetable foods exposed to heat and light does not depend upon the carotene itself or on the presence of oxygen, primarily, but is conditioned by the presence of other substances.

Carotene is also decolorized when it is transformed to vitamin A in the animal body. While this change apparently involves the loss of prooxidant activity (18) the analogy ceases here. Whatever the change, it is such as to leave intact that portion of the molecule which is concerned in its physiological action. That structure still retains its instability toward heat, ultra-violet radiation, and oxygen.

An explanation of the unusual physiological and chemical

properties of carotene must await further information on the structure of the molecule and on the probable electronic alterations which it may undergo. Unfortunately a lack of material has prevented a detailed elaboration of some of the elementary facts presented in this paper.

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SUMMARY

1. Crystalline carotene was obtained from the unsaponifiable lipids of lettuce.

2. Carotene crystallizes in the hexagonal system.

3. The fading of the crystals at elevated temperature is not an oxidation.

4. Carotene solutions may be bleached by heat, ultra-violet light, and by the presence of autoxidizable fats.

5. The presence of hydroquinone delays for a variable length of time the bleaching of carotene solutions by any of the above mechanisms.

6. Hydroquinone protects ethyl laurate solutions of carotene apparently indefinitely. Such solutions are satisfactory for physiological studies.

7. In autoxidizable mixtures, carotene is an active prooxidant. This property is unique in a hydrocarbon.

8. The physiological activity of carotene as vitamin A is confirmed; under widely differing conditions, the growth induced by feeding carotene seems to be directly proportional to the amount fed; 0.005 mg. permits an increment of 3 to 5 gm. in the weight of rats deprived of vitamin A.

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FURTHER STUDIES OF THE EFFECT OF INSULIN ON THE AMINO ACID CONTENT OF BLOOD

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INTRODUCTION

During the past year we have had occasion to investigate anew the effect of insulin on the amino acid content of blood. Although several contributors to the subject have reported in carefully conducted experiments that insulin lowers the blood amino acids of diabetic and normal subjects, there are two indications that this reduction may have been due not to the insulin but to the mere withdrawal of blood. Von Falkenhausen and Boehm (1) have stated that the collection of 1 to 3 cc. of blood by simple venous puncture is followed within 10 minutes by a hypoaminoacidemia of 1 to 3 mg. per cent. Likewise Bischoff and Long (2) reported that the reduction in the blood amino acids of insulinized rabbits was no greater than in control animals which received no insulin. With respect to these statements it is to be recognized that they lead to conclusions which can be valid only in restricted cases and under quite limited conditions. The observations of Rosenbaum (3) and Wiechmann and Dominick (4) do not confirm the statement of von Falkenhausen and Boehm, nor have we, in the course of this investigation, found any indication of hypoaminoacidemia in control human subjects. In rabbits the amino nitrogen content of the blood is apparently subject to greater fluctuations. Although in the experiments reported by Luck, Morrison, and Wilbur (5) the control animals showed little if any hypoaminoacidemia we have more recently had opportunity to observe that the withdrawal of blood samples of large size (3 to 5 cc. each) frequently leads to a reduction of the amino nitrogen content. On the assumption that this decrease in control animals might be

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due to the severity of the hemorrhage (10 to 15 per cent of the total blood having been removed) we have turned to human subjects from whom six or seven large samples could be taken without sacrificing more than 2 per cent of the total blood volume.

In addition to this consideration the contributions to the subject have been surprisingly scanty and have rendered increasingly necessary a further investigation of the problem. Although there is a marked degree of unanimity in the conclusions that have been reached by investigators, it is clear that in most instances the number of subjects studied has been too small. Occasionally the observations were incidental to the study of another problem. Bickel and Collazo who were the first to report that insulin reduces the amino acid content of blood ("stark der Norm genähert") were studying vitamin B avitaminosis in pigeons and did not incorporate their data in the paper (6). Wolpe (7), whose observations led him to the same conclusion, published the experimental findings from only one subject, a diabetic. Tashiro's conclusions (8), similar in nature, are based on experiments upon only two rabbits. Silberstein, Rappaport, and Wachstein (9) pointed out that insulin administered to depancreatized dogs always lowered the amino nitrogen content of the blood ("stets eine deutliche Herabsetzung"), minimum values being reached within 2 to 5 hours, but did not publish the supporting data. Likewise Bufano (10) gave insulin alone to but one dog and observed a marked reduction in the amino nitrogen content of the blood. So also Wiechmann and Dominick (4), after experiments on but two diabetic subjects, concluded that insulin accelerated the disappearance of intravenously injected glycine.

Procedure

The subjects chosen were male students in Stanford University, mostly from the first and second year classes in medicine. In each case breakfast was omitted on the day of the experiment. At the commencement (usually 7 a.m.) the subject drank 200 cc. of water. 1 hour later the first blood sample was drawn. Insulin was administered 15 to 30 minutes later by subcutaneous injection in the forearm. Five other blood samples were drawn at intervals of $\frac{1}{2}$, $1\frac{1}{2}$, $2\frac{1}{2}$, 4, and 5 hours after administration of the insulin. Neither water nor food was received by the subject until the conclusion of the experiment. The seven subjects who

served as controls followed the same routine but received 0.6 cc. of 0.9 per cent sodium chloride instead of insulin.

25 units of insulin, approximately 0.3 units per kilo, were administered to each of the experimental subjects. This quantity was contained in 0.63 cc. of the preparation employed (Lilly's iletin, 40 units per cc.).

The blood was collected over a trace of dry potassium oxalate. Each sample was of 13 cc., a volume which permitted the determination of amino acid nitrogen, reducing sugar, and hemoglobin. Amino acid nitrogen was determined in every sample by both colorimetric and volumetric methods. The latter procedure was a modification of that described by Luck (11). 10 cc. of blood were run into 35 cc. of boiling 0.01 N acetic acid contained in a large tube. After 2 minutes the tube was removed from the bath and cooled. 3 cc. of 50 per cent trichloroacetic acid were added, the contents were diluted with water to 50 cc., and the volume checked by weighing. The contents were thoroughly mixed, treated with infusorial earth, permitted to stand 30 minutes for complete protein precipitation, and then filtered. An aliquot portion of the filtrate was evaporated, freed of ammonia, and analyzed as described previously (11).

The method of Folin (12) was employed for the colorimetric determination of amino acid nitrogen. Reducing sugar was estimated by the method of Folin (13), and hemoglobin by the acid hematin method of Sahli. The first sample in each experiment served as a standard for the hemoglobin determinations. To it was arbitrarily assigned the value 100. The results are summarized in Tables I and II.

DISCUSSION

It is apparent from Tables I and II that a definite lowering in the amino nitrogen content of the blood is to be observed in the insulinized subjects.[1] At the same time the constancy of the amino

[1] As may be expected, the colorimetric and volumetric methods did not agree quantitatively although qualitative agreement is clearly indicated. We are satisfied that a major cause of the discrepancy is in the use of tungstic acid in the one method and heat coagulation followed by trichloroacetic acid in the other. Differences in the degree of protein precipitation and amino acid adsorption are probable. The desirability of further research into the chemistry of protein precipitation by the alkaloidal reagents is apparent.

TABLE I

Effect of Insulin on Blood Amino Acids, Sugar, and Hemoglobin

	Time after injec- tion	R-N	S-P	P-R	S-H	C-Y	S-N	B-K	Cr-R	M-N	W-R*
	hrs.										
Amino acid N,	0	6.8	5.3	7.7	4.3	6.4	7.1	6.1	7.5	5.5	6.2
volumetric	0.5	5.6	4.9	7.3	3.4	5.7	6.5	5.8	7.3	5.1	6.4
method, *mg.*	1.5	5.2	4.9	6.4	3.6	5.3	6.3	5.6	6.2	5.4	6.9
per 100 cc.	2.5	4.9	4.8	5.7	3.1	5.5	4.8	5.2	6.5	4.6	6.4
	4.0	5.0	4.5	5.5	2.6	5.8	4.6	4.8	6.6	4.4	5.6
	5.0	4.8	4.4	5.3	3.4	6.4	4.5	4.6	6.1	4.1	5.3
Amino acid N,	0	5.3	5.9	8.7	7.2	4.2	5.5	4.7	4.3	6.6	4.7
colorimetric	0.5	4.7	5.5	8.7	7.0	3.8	4.3	4.7	4.6	5.2	4.9
method, *mg.*	1.5	4.4	5.5	8.0	6.7	3.7	4.1	4.5	4.1	4.9	5.2
per 100 cc.	2.5	4.8	5.1	7.7	7.0	3.8	3.9	4.4	3.9	4.1	4.5
	4.0	4.9	4.7	6.7	5.8	3.8	3.7	3.8	3.6	4.1	4.2
	5.0	4.9	4.3	6.0	4.1	4.0	3.5	3.5	3.7	4.0	3.8
Reducing	0	84	89	84	95	80	89	88	96	104	60
sugar, *mg.*	0.5	54	35	50		55	65	68	76	83	31
per 100 cc.	1.5	58	50	46	81	54	62	65	54	68	45
	2.5	51	71	50	58	50	59	58	52	59	40
	4.0	90	71	48	60	49	56	56	46	55	40
	5.0	53	61	56	65	45	48	51	47	59	38
Hemoglobin,	0					100	100	100	100	100	100
per cent	0.5					99	101	97	77	104	
	1.5					101	102	99	78	105	83
	2.5					101	100	96	79	102	78
	4.0					101	99	96	79	99	78
	5.0					103	99	98	78	100	78

Each subject received 25 cc. of insulin.

R-N.   200 cc. of water taken prior to the fifth sample. Profuse sweating.

S-P.   Sweating for ½ hour 3 hours after insulin. Hand tremors 1 hour after insulin.

P-R.   Sweating 3 hours after insulin.

S-H.   Tremors in hand 2 to 2½ hours after insulin. Sweating 3 hours later.

C-Y.   No symptoms.

S-N.   Mild sweating 1½ hours after insulin. Tremors in hands and arms.

B-K.   Sweating 1½ hours after insulin. Muscle tremors.

Cr-R.   No symptoms.

TABLE I—*Concluded*

M-N. General lassitude.

W-R. Muscle tremors and facial pallor ½ hour after insulin. Blurred vision in left field 1½ hours after insulin.

* This subject proved to be of unusual interest. ½ hour after the injection of insulin, when the second blood sample was being drawn, marked muscle tremors and premonitory signs of fainting suddenly became evident. Within 2 minutes muscular control and full consciousness were restored. A blurring of vision was experienced 2 hours later. Completion of the blood sugar determinations indicated that the subject possessed a fasting blood sugar value about two-thirds of that which is considered normal. In consequence even the small amount of insulin administered was sufficient to lower the blood sugar to the convulsive level. The unusual behavior of the amino acids and hemoglobin also indicates that the subject is to be considered abnormal.

nitrogen values in the control subjects demonstrates that the hypoaminoacidemia cannot be attributed simply to withdrawal of blood. We are therefore satisfied that these results confirm the observations of Luck, Morrison, and Wilbur (5) and support also the recent work of Kerr and Krikorian (14).

We are somewhat at a loss, however, to explain by any single theory this effect of insulin on protein metabolism. Nevertheless, several deductions may properly be made.

First of all, it is clear that the reduction in amino nitrogen content is not due to mere dilution of the blood. The hemoglobin values indicate, so we feel, that there is but little passage of tissue fluid into the blood.

Furthermore, there is no lowering of the renal threshold for amino acids. This is demonstrated by the work of Wiechmann (15) and Goffin, Rahier, and Regnier (16) and by several observations in this research on the effect of insulin on amino acid excretion. In fact all the evidence points to a reduction in the concentration of urinary amino acid and in the rate of excretion.

Again, as Kiech and Luck have reported (17), insulin hypoaminoacidemia is not due to a simple increase in the rate of absorption of amino acids by the organs. At least if there is any increase in the hepatic and peripheral utilization of amino acids, it is not indicated by the amino nitrogen content of the liver and muscle, or by analysis of the whole carcass. Actually there is a general reduction in the free amino acid content of the entire animal.

It is of course apparent that an increased tissue absorption of amino acids may actually take place, but by virtue of an associated increase in protein or peptide synthesis, no accumulation

TABLE II

Control Results and Final Averages

Control subjects received 1 cc. of 0.9 per cent NaCl.

	Time after injection	G-R	T-N	H-W	K-H	D-Y	C-R	H-T	Averages	
									Controls	Insulin subjects
	hrs.									
Amino acid N,	0	5.0	5.9	3.6	6.6	6.2	7.3	5.7	5.8	6.3
volumetric	0.5		5.8	3.5				5.5		5.8
method, *mg.*	1.5	4.9	5.8	3.4	6.5	6.3	7.3	5.7	5.7	5.6
per 100 cc.	2.5	4.8	6.1		6.6	6.2	7.3	5.6	6.1	5.2
	4.0	4.9	5.9	3.5	6.5	6.3	7.5	5.6	5.8	4.9
	5.0		5.7	3.5	6.6	6.3	7.3		5.9	4.9
Amino acid N,	0	5.2	4.9	4.0	5.8	6.7	7.2	5.8	5.7	5.7
colorimetric	0.5	5.2	4.9	3.9	6.0	6.7	7.3	5.8	5.7	5.3
method, *mg.*	1.5	5.2	4.8	3.9	5.5	6.8	7.3	5.7	5.6	5.1
per 100 cc.	2.5	5.2	4.8	4.0	5.9	7.0	7.2	5.7	5.7	4.9
	4.0	5.2	4.8	4.0	5.5	6.7	7.1	5.7	5.6	4.5
	5.0	5.1	4.8	4.0	5.4	6.7	7.2	5.7	5.6	4.2
Reducing sugar,	0	88	99	97			102	87	95	87
mg. per 100 cc.	0.5	77	96	98			100	88	92	57
	1.5	77	95	95			99	86	90	58
	2.5	82	98	98			100	86	93	55
	4.0	80	97	99			100	86	92	57
	5.0	83	96	97			101	86	93	52
Hemoglobin, *per*	0	100	100	100	100	100	100	100	100	100
cent	0.5	97	99	98	100	80	97	96	97	96
	1.5	95	94	98	100	89	99	82	94	95
	2.5	98	103	97	102	89	98	98	98	93
	4.0	95	100	97	102	95	98	100	98	92
	5.0	97	101	98	100	92	100	95	98	93

of free amino acids would be observed. This interesting possibility, suggested by Wiechmann (18), arises by analogy from the well recognized transformation of glucose to glycogen in the insu-

linized animal and the attendant reduction in blood and tissue reducing sugar. Unfortunately, through lack of satisfactory methods for investigating protein storage, this hypothesis has received inadequate study.

Others have considered (17, 19) that insulin may inhibit amino acid formation from peptides and tissue proteins. It is, however, clear that in some respects this hypothesis differs but little from the preceding one and is equally difficult to inquire into experimentally.

Again it is not permissible to regard the reduction in amino acid content as secondary to the hypoglycemia. The work of Luck and Spaulding (20) on rats and that of Ralli and Tiber (21) on depancreatized dogs show that the simultaneous administration of glucose does not prevent the insulin hypoaminoacidemia and fall in tissue amino acid nitrogen.

To some extent this fact would seem to discredit the hypothesis that insulin hypoaminoacidemia is due to an increased rate of glucogenesis from amino acids under conditions in which the increased catabolism of amino acids fails to be compensated by an equal increase in the liberation of amino acids by tissue protein hydrolysis. For as usually expressed (22, 23) the hypoglycemic state is regarded as a stimulus to glucogenesis. Nevertheless, the increase in urea formation induced by insulin (14, 17, 24) and the increased breakdown of hepatic protein reported by Paschkis (23) are best explained by postulating an increased rate of protein catabolism. To reconcile these various findings it is necessary to abandon the concept that the hypoglycemic state is the principal stimulus and to regard the action of insulin on protein metabolism as independent of its hypoglycemic action in carbohydrate metabolism.

We consider the most acceptable theory to be that insulin accelerates the catabolism of amino acids without a compensatory increase in the regeneration of amino acids from tissue proteins. The possibility of an accompanying increase in the peripheral synthesis of protein from the blood and tissue amino acids is not incompatible with the experimental findings. The effects are not secondary to hypoglycemia.

SUMMARY

1. Insulin was administered by subcutaneous injection to ten adult human subjects. Seven others, who served as controls, received injections of 0.9 per cent sodium chloride.

2. The observations of Luck, Morrison, and Wilbur on the reduction of the amino acid concentration in the blood of insulinized subjects were confirmed.

3. The mode of action of insulin is discussed.

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THE ASSOCIATION OF VITAMIN A WITH GREENNESS IN PLANT TISSUE

III. VITAMIN A CONTENT OF ASPARAGUS GROWN UNDER LIGHT OF VARIOUS QUALITIES*

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INTRODUCTION

In two former publications (1, 2), the authors submitted data which led to the conclusion that a relationship appears to exist between the vitamin A content of plant tissues (lettuce and aspara-gus) and their degree of greenness. Some consideration was given also to the influence of variations in the chemical composition of the tissues.

In the meantime, McLaughlin, in cooperation with Haber, has reported (3) on the relation of vitamin A content to size of leaves, as found with the so called New Zealand spinach plant. Their experimental results confirmed their hypotheses that if the more actively functioning cells elaborate vitamin A more rapidly, and if it is elaborated only in chlorophyll-bearing cells, then young leaves should be more potent than older ones and thin leaves of greater surface area should be superior to thicker leaves from the same plants. Since greater proportional surface area of leaves means more superficial cells in which the chloroplasts are more concentrated than in deeper lying cells (quantitative chlorophyll determinations were not reported), their findings give support to the theory of interdependence between chlorophyll development and vitamin A content.

Heller's (4) results with seedlings produced under conditions

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* Published from the Michigan State College Agricultural Experiment Station as Paper 50, new series.

of various intensities and qualities of light also support the relationship mentioned above. Clearly, the vitamin A content coördinated with the properties of the light used for illumination of the plants. The amount of light received by leaves depends directly on their surface area; where the light is such that the chlorophyll will develop, leaves of greater surface area are likely to be higher in chlorophyll, and hence, the relationship becomes a triple one which involves light, chlorophyll, and the vitamin.

On the other hand, Morgan and Smith (5), and also House, Nelson, and Haber (6) have shown the green, unripened fruits of the tomato plant to be relatively poor in vitamin A as compared with that for the fruits ripened either on the plant or by some three or four more or less artificial methods. This information regarding fruits is interesting and valuable; the fruit being both structurally and physiologically different from the leaf.

Since in the experiment of Morgan and Smith, the fruits were ground (the green ones as soon as picked, the other lots at the ends of the periods of time required for ripening), stored in airtight jars under an atmosphere of carbon dioxide at a low, freezing temperature, and the number of animals used was small, the experiment of House, Nelson, and Haber has a more direct relation to the work of the present authors. Their results, when the animals were fed 2 gm. of material daily from the fresh fruits, indicate a good supply of vitamin A in the green fruits, in fact, the animals so fed maintained a higher average rate of growth from the 1st to the 5th week than those of any other lot. Thereafter, this rate declined, while the others did not. This suggests the need for investigation of the complex phenomena of fruit ripening with respect to their influence on the vitamin. At present, it is difficult to conceive of added quantities of the vitamin being synthesized after the decline and disappearance of the chlorophyll.

The next logical step in our investigations appeared to be that of producing plants which would vary gradually in chlorophyll content from zero to a maximum quantity and to determine their respective vitamin A efficiencies by the use of test animals. Asparagus was chosen as the plant best adapted for the purpose. A preliminary experiment conducted in 1928, with varicolored and uncolored light filters, gave every indication of the possibility of employing such a method to secure asparagus tips of variable

greenness whose vitamin A properties as food for test animals would correlate with their degrees of greenness. Accordingly, specially manufactured filters were obtained and used experimentally during the 1929 and 1930 seasons.

Methods

The asparagus plants were the same as used in the previous experiments (2). Light filters, each 6 by 6 inches, were supplied by the Corning Glass Works. Twelve filters of each type were set in putty on narrow metal bars in three rows in a sash which was hinged on a light-tight wooden frame, a strip of felt intervening between the sash and the edge of the frame. This frame, which was 30 inches long, 24 inches wide, 9 inches high at one end

TABLE I

Transmission Properties of Glass Filters

Filter	Limits of transmission	Transmission of equal energy spectrum
	mμ	*per cent*
G 586 A	320–420	10.7
G 55 A	325–580	20.1
G 24	601–infra red	18.3
Vita glass	258–700+	88.0*

* Total energy of sunlight transmitted.

and 7 inches at the other, and furnished with light-tight ventilation, was set over the asparagus plants in the garden row and anchored there. Three such frames, holding filters designated by the manufacturer as G 586 A red-purple, G 55 A signal purple, G 24 red, were used. The same kinds of filters were among those used successfully by Sayre (7) in studying the relationship of wavelengths of light to chlorophyll development in seedlings of various species. Data, taken from Sayre's paper, on the transmission of the filters are given in Table I. In addition, a fourth frame covered by a sash of Vita glass was used, and, besides this, lots of animals were fed from tips (*a*) grown in the open, and (*b*) bleached by covering the rows with soil and taking the tips before they reached the surface of the ground.

The laboratory technique did not vary essentially from that

employed formerly (2). Albino rats were placed on a vitamin A-free ration, complete in all other dietary essentials, until their store of the vitamin was depleted. Thereupon, they were fed daily a given amount per animal of the asparagus (taken from the region just below the terminal bud of the stalk) and their growth determined over a period of 8 weeks.   This was in the 1929 season. The daily supply of tips from the frames was too limited to permit the taking of samples for other purposes.

In 1930, the frames were put out again, and the tips produced over an 8 week period used exclusively for quantitative determinations of chlorophyll, amino acids, water-soluble nitrogen, and inorganic elements.

In the chlorophyll determinations, only tips which had been above ground the same length of time and thus had had equal exposure to the light in the frames were taken as samples, placed in light-proof bags, and brought to the laboratory.   A 2 to 3 inch section was taken from below the terminal of each tip; these sections were cut into small pieces, and the whole mixed together. From this mass a weighed quantity was taken and carried through the process of extraction, etc., according to the method employed by Schertz (8).   The standard solution for use with the Duboscq colorimeter was made up from a sample of purified chlorophyll $\alpha + \beta$ which Dr. F. M. Schertz of the United States Department of Agriculture was kind enough to furnish.

The sample for determination of soluble nitrogen and amino nitrogen was taken and made ready in the same manner as that for chlorophyll determination.   After thorough maceration and extraction with water, and clarification by centrifuging and filtering, amino acids were determined on aliquots out of a standard volume by the Van Slyke method, and water-soluble nitrogen by the Kjeldahl method.

The quantitative determinations of inorganic elements were made by the Experiment Station chemist, using standard methods of procedure (2).   The samples analyzed thus for inorganic constituents were composites of tips gathered over the period as they were available.   After having been washed, and cut into small pieces, they were first dried by an electric fan and then dried gradually to 95°, beginning at 50°, in an electric oven, and then ground to a 60 mesh sieve.

Results

The data, in condensed form, are given in Table II. The figures for chlorophyll, water-soluble nitrogen, and amino nitrogen are averages, each for six determinations made at intervals from May 11 to June 24. Those for the inorganic elements are from single determinations made on samples acquired as mentioned in the section on methods. The amount of asparagus tissue fed daily per animal was 0.1 gm.

The primary aim of the experimental procedure was the pro-

TABLE II

Data on Chemical Composition of Asparagus Tips and Gains of Albino Rats

Glass	No. of animals	Survivals	Average gain	Chlorophyll in 10 gm. fresh tissue	Water-soluble N	Amino N	Ratio, water-soluble N:amino N	Inorganic elements in per cent of dry weight				
								Ca	Fe	Mn	P	K
			gm.	*mg.*	*per cent*	*per cent*						
None (bleached)	3	0*	−5.42	0	4.49	1.61	2.79	0.239	0.014	Trace	0.753	3.60
G 586 A	8	6	−0.11	0.063	4.54	1.39	3.30	0.150	0.017	0.001	0.717	3.32
G 55 A	8	8	+2.39	0.088	5.54	1.98	2.81	0.176	0.014	Trace	0.774	3.34
G 24	9	9	+2.67	0.181	4.45	1.39	3.19	0.207	0.013	"	0.738	3.18
Vita	10	10	+3.71	0.423	6.12	1.74	3.45	0.223	0.017	"	0.749	3.02
None (open)	9	8	+2.10	0.479	5.80	1.52	3.89	0.264	0.013	0.002	0.863	3.52

* None alive at end of 6th week.

duction of tips that would vary significantly in chlorophyll content. The achievement of this purpose is certified by the fifth column of Table II. Secondly, it was hoped that differences in other properties, which might have at least a conceivable association with the synthesis and quantity of vitamin A should develop in order that these might be both united and compared with the chlorophyll content as either accessory or else deciding factors. This too was realized.

Table II gives some indication that with increasing water-soluble nitrogen and its ratio to amino nitrogen, increasing calcium content, and decreasing potassium content the vitamin A effective-

ness of the tissue also increased. However, a close analysis of any one of these apparent relationships reveals discrepancies sufficient to render its reality extremely doubtful. The relationship between chlorophyll content and the vitaminic property of
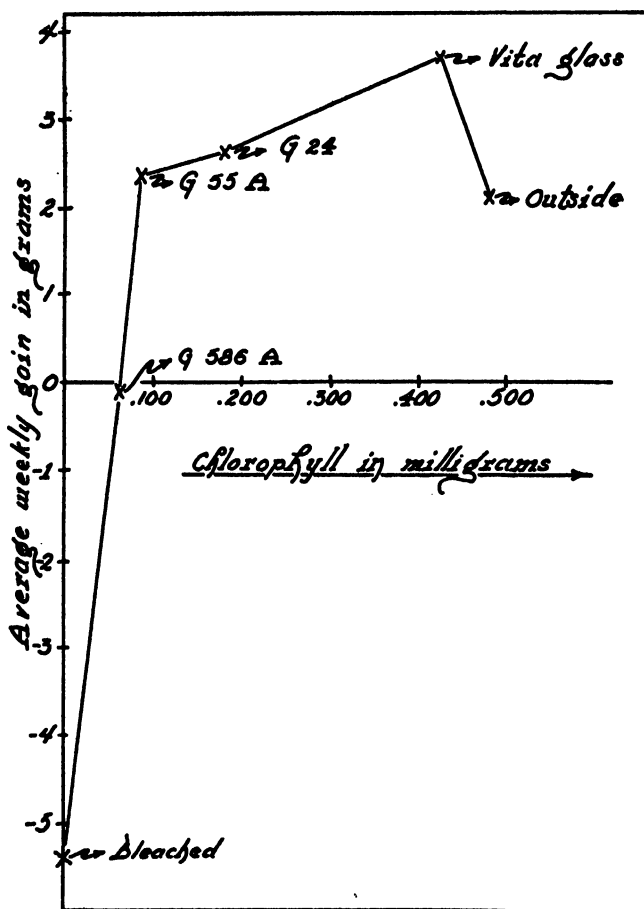

Fig. 1. Relation of chlorophyll to animal growth

the tips is the most systematic and conclusive, in fact, the only one whose graph shows acceptable uniformity over its entire range. See Fig. 1.

Fig. 1 shows plainly that a relationship exists between the two variables, and that it is not linear in character. Beyond the

point G 55 A the growth of the animals in proportion to chlorphyll content lessens and finally drops sharply with respect to the asparagus grown in the open. This course of the curve could be interpreted as a progressive diminution of the dominance of chlorophyll as the limiting factor, and, at last, the ascendancy of other factors as limiting, when chlorophyll is fully ample and no longer the limiting factor. The environal conditions (data taken but not presented) under which the tips in the open grew were much different from those which obtained in the closed frames. Besides the full sunlight outside, both the temperature and relative humidity averaged considerably lower.

The possibility that light in itself, with respect to its quality in particular, may more or less directly (aside from its influence on chlorophyll formation) determine the production and strength of the vitamin cannot be overlooked. Morton and Heilbron (9) have found that certain fish liver oils and vitamin A concentrates show a conspicuous absorption band at 328.5 $m\mu$ and that the intensity of this band closely parallels the vitamin A potencies of these substances. This, however, need not, as might be supposed, mean that light containing wave-lengths of that order is indispensable for the production of the vitamin in plants. Its presence is not essential to chlorophyll formation. The asparagus tips grown under glass G 24 (Table I) were deprived of all light of wave-length less than 601 $m\mu$ and yet were higher in chlorophyll and also in vitamin A than those grown under either G 55 A or G 586 A which admitted the said wave-length of light.

In general agreement with the conception of a quantitative association between vitamin A and chlorophyll, Bezssonoff (10) has reported that plant leaves are as much richer in vitamin A as they have greater abundance of chlorophyll. He has studied the vitamin from the standpoint of its rôle in the plant where it is produced. His data have led him to classify the vitamin as a lipid substance which accompanies chlorophyll and can be freed from it. He holds vitamin A to be a sensitizer of chlorophyll, and hence a conditioner of chlorophyll formation. The present authors, who think the reverse of this is true, are unwilling to concede the point prior to a time when plant tissue which is entirely devoid of chlorophyll and yet abundant in vitamin A shall have been realized and its vitaminic potency demonstrated in terms of adequate gains in weight by animals fed thereon.

CONCLUSION

The experimental evidence presented here gives added support to the conception that in some manner or other the elaboration of vitamin A in the plant is connected with the development of the chlorophyll pigment. For the first time, a reasonably successful attempt to establish this association on a quantitative basis has been made. It seems reasonable to conclude that within the restrictions of the two variables expressed by some non-linear relationship chlorophyll content is a limiting factor on vitamin A synthesis in the vegetative parts (in this case the stem tip) of the plant. This gives point to the contention of Schertz (11) that further progress in a knowledge of vitamin A may depend much upon the industry and the success of the biochemists and the plant physiologists in their efforts to solve the mysteries of chlorophyll and its functions.

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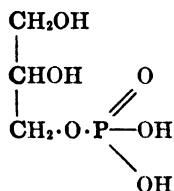11. Schertz, F. M., *Science*, **68**, 48 (1928).

THE RATE OF HYDROLYSIS OF α- AND β-GLYCERO-PHOSPHATES BY ENZYMES
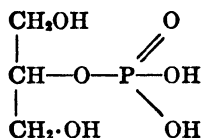
By H. D. KAY and E. R. LEE

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It is well known that a glycerophosphoric acid may have one of two isomeric forms.

Of these the α form (I) can display optical activity, but the β form (II) cannot. Commercial preparations vary from almost pure α-, through mixtures of varying proportions of α- and β- to almost pure β-glycerophosphate.

During the last few years the enzymic hydrolysis of glycerophosphate has been the subject of several communications (1–3), but unfortunately, in most of these papers the authors do not state whether they employed the (racemic) α or the β form. This would appear to be an omission of some importance when detailed results with regard to comparative kinetics, effect of activators, etc. are being reported, and one of us (4) found that the phosphatase from mammalian kidney attacks synthetic β-glycerophosphate more readily than the *dl,α* form in the region of the optimum pH for this enzyme and these substrates (8.8 to 9.2). On the other hand Fleury and Sutu (1) state that an enzyme from white mustard, and Karrer and Freuler (3) that phosphatase from the hepato-pancreas of the vineyard snail, hydrolyze both synthetic α- and β-glycerophosphates at the same rate. Kay's finding is much less surprising than that an enzyme should hydrolyze both substrates

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equally rapidly. Moreover, it has been stated by Akasawa (2) that the optimal pH for hydrolysis by taka-phosphatase is not the same for the two isomers.

Recently, owing to the kindness of Dr. F. L. Pyman, who provided us with authentic specimens of salts of α- and β-glycerophosphoric acids,[1] prepared synthetically by the methods of King and Pyman (5) we have been able to examine this point more closely and to show definitely that both animal and plant phosphatases attack the two isomers—the racemic α- and the β-glycerophosphate—at different rates, and that the different rates at which they undergo enzymic hydrolysis can be made use of for distinguishing between the two isomers.

<div align="center">EXPERIMENTAL</div>

Substrates—The synthetic salts, received in the form of sodium β-glycerophosphate and calcium α-glycerophosphate were both converted into the barium salts, and then, for the enzymic hydrolysis, into either the sodium or potassium salts by adding a solution of the appropriate sulfate. The dilute substrate solutions, containing racemic α- or β-glycerophosphate, after adjusting to the optimum pH of the enzyme to be employed, were further diluted to contain the same amount of P per cc.

Enzyme Solutions—The following were used: taka-phosphatase, soy bean phosphatase, kidney phosphatase, plasma phosphatase.

Conditions for Hydrolysis—The hydrolyses were usually carried out over a range of pH which covered the optimum for each enzyme preparation. The buffering was done in the alkaline range (for kidney or intestinal phosphatase) by Sørensen's glycine-NaOH buffers, and by phthalate buffers in the acid (taka-phosphatase or soy bean phosphatase) range. In all cases the amount of hydrolysis represented only a small fraction of the total substrate present.

Effect of Different Enzymes on Pure α- and β-Glycerophosphates

(a) *Taka-Phosphatase*—The optimum pH for taka-phosphatase[2] determined in preliminary experiments was found to be between

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[1] Sodium β-glycerophosphate may now be obtained from the Research Chemicals Department of Boot's Pure Drug Company, Nottingham, England.

[2] In taka-diastase (Parke, Davis and Company).

3.4 and 3.8 for β-glycerophosphate, but was a broad one stretching from 3.4 to 6 or beyond with the synthetic α isomer.

The rates of hydrolysis by this enzyme of pure α-glycerophosphate and pure β-glycerophosphate and mixtures of the two salts
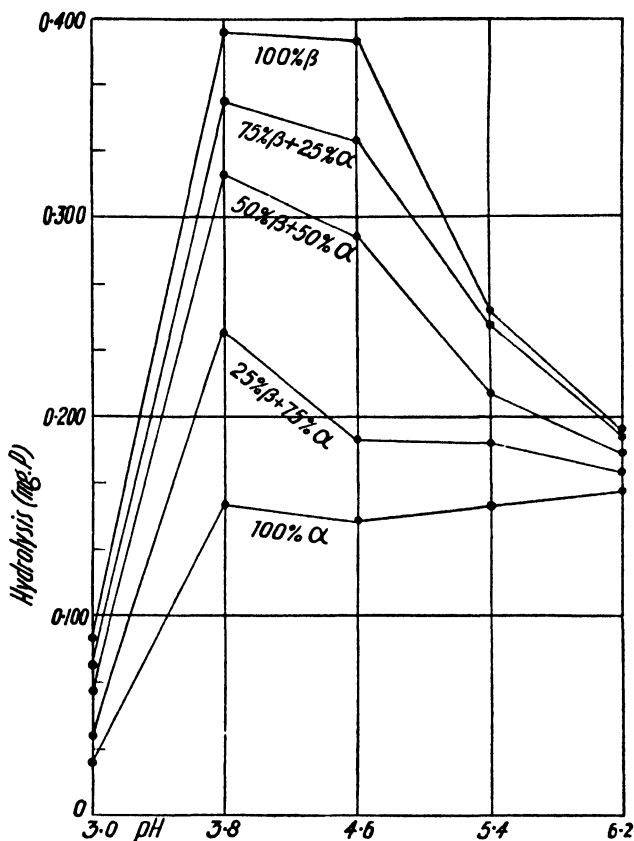
FIG. 1. Rates of hydrolysis by taka-phosphatase of pure Na α-glycerophosphate and Na β-glycerophosphate and mixtures of the two salts in varying proportions, at pH near the optimum.

in varying proportions, are shown in Fig. 1. The pure α and β esters are hydrolyzed at markedly different speeds.

It would appear from this figure that the pH-activity curves for the action of taka-phosphatase on pure α- and pure β-glycerophosphates are quite characteristic for the two esters, and that a

rough estimate is also possible of the composition of any mixture of synthetic α- and β-glycerophosphates by comparing the pH-activity curve of such a mixture with that given by a series of known mixtures such as those shown in Fig. 1.   It is, of course, necessary to carry out the hydrolyses of any given series at the same time and with the same enzyme under strictly standardized conditions.

(b) *Soy Bean Phosphatase*—The optimum pH determined approximately in a previous experiment was found to be about 5.2.   With phthalate buffers extending over a short range on each side of this pH, the activity of a filtered aqueous extract of macer-

TABLE I

Hydrolysis of α- and β-Glycerophosphate by Soy Bean Phosphatase

Hydrolysis results are given in mg. of P per 10 cc. of reaction mixture in 2 hours.

pH	Sodium α-glycerophosphate	Sodium β-glycerophosphate
3.0	0.041	0.039
3.4	0.084	0.085
3.8	0.145	0.146
4.2	0.206	0.207
4.6	0.257	0.256
5.0	0.286	0.299
5.4	**0.299**	**0.326**
5.8	0.286	0.317
6.2	0.257	0.288
7.0	0.167	0 191

ated soy beans was determined from pH 3.0 to 7.0, using both sodium α- and sodium β-glycerophosphate as substrates, under strictly comparable conditions.   Adequate controls were run at the same time.

It will be observed (Table I) that at the optimum pH of hydrolysis by soy bean phosphatase, β-glycerophosphate is rather more actively attacked than the racemic α-glycerophosphate, though the difference in rate of hydrolysis is much less than that observed with phosphatase from other sources.

(c) *Kidney Phosphatase*—Kidney phosphatase in the region of optimum hydrogen ion concentration for its activity on glycerophosphate (pH 8.9 to 9.2) hydrolyzes the β ester distinctly faster

than the α under similar conditions. This is shown, *inter alia*, in Fig. 2 and Fig. 3, confirming earlier findings (4).

(d) *Plasma Phosphatase*—As has been already described by one of us (6), α- and β-glycerophosphates are not hydrolyzed at the same rates by plasma phosphatase. At pH 7.6 the enzyme hydrolyzes the synthetic α form about twice as fast as the β ester. At pH 8.9, however, the plasma enzyme hydrolyses the β ester
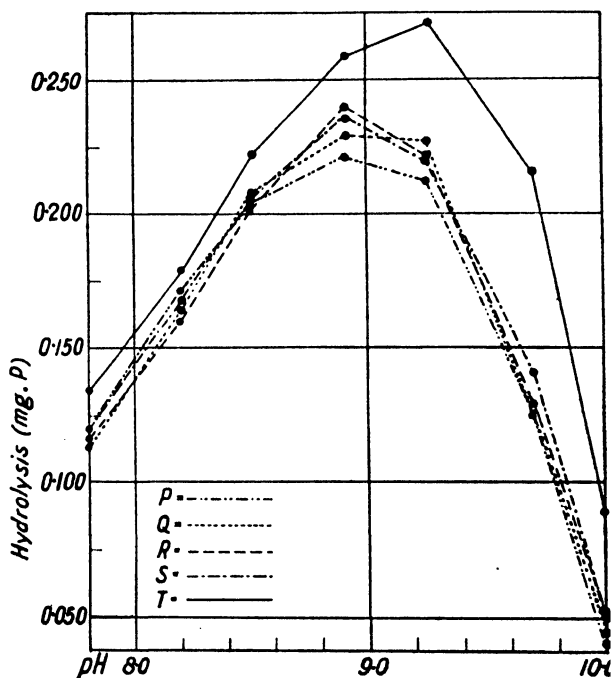
Fig. 2. Rates of hydrolysis by kidney phosphatase of pure Na α-glycerophosphate (P), pure Na β-glycerophosphate (T), and three fractions of enzymically synthesized Na glycerophosphate (Q, R, S) at pH near the optimum.

faster than the α form, as with kidney phosphatase. The effect of Mg ions (*vide infra*) on the rate of these reactions was to accentuate this difference. Thus in one experiment, at pH 8.9, in absence of added Mg the amount of hydrolysis by the same quantity of enzyme in the same time was, for the α substrate 0.113 mg. of P, for the β, 0.130 mg. of P; in presence of 0.004 M MgCl$_2$ the rates were

0.41 mg. of P for the α- and 0.60 mg. of P for the β-glycerophosphate. The effect of this addition of Mg is thus to increase the initial rate of hydrolysis from three to five times.
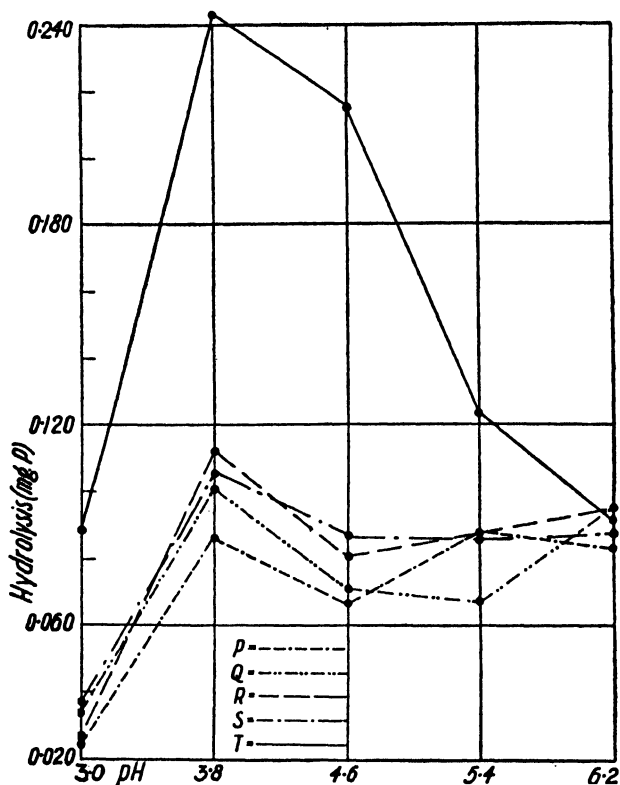
FIG. 3. Rates of hydrolysis by taka-phosphatase of pure Na α-glycerophosphate (P), pure Na β-glycerophosphate (T), and three fractions of enzymically synthesized Na glycerophosphate (Q, R, S) at pH near the optimum.

Effect of Mg Ions

(a) *Taka-Phosphatase*—Magnesium ions apparently inhibit somewhat the hydrolysis of β-, but accelerate that of racemic α-glycerophosphate by taka-phosphatase (phthalate buffers) (see Tables II and III).

(b) *Dialyzed Kidney Phosphatase*—Magnesium ions markedly

accelerate the hydrolysis of both dl,α- and β-glycerophosphate by dialyzed kidney phosphatase (glycine buffers) (see Table IV).

Use of This Method of Discrimination

Two examples may be given.

1. *Identification of an Unknown Preparation by Comparison of Its Rate of Hydrolysis by Enzymes with That of Authentic α- and*

TABLE II

Effect of Mg Ions on Hydrolysis of α- and β-Glycerophosphate by Taka-Phosphatase

Hydrolysis results are given in mg. of P per 10 cc. of reaction mixture.

pH	Na β-glycerophosphate		Na α-glycerophosphate	
	Without Mg	With Mg (0.004 M)	Without Mg	With Mg (0.004 M)
3.0	0.353	0.162	0.159	0.149
3.4	0.442	0.351		0.176
3.8	0.466	0.380	0.146	0.232
4.2	0.451	0.378	0.204	0.256
4.6	0.406	0.372	0.216	0.232

TABLE III

Comparison of Unknown Glycerophosphate (X) with Authentic α- and β-Glycerophosphates, Using Taka-Phosphatase

Hydrolysis results are given in mg. of P per 10 cc. of reaction mixture.

pH	Authentic β-glycerophosphate		Glycerophosphate X		Authentic α-glycerophosphate	
	Without Mg	With Mg (0.004 M)	Without Mg	With Mg (0.004 M)	Without Mg	With Mg (0.004 M)
3.0	0.44	0.45	0.44	0.49	0.28	0.30
3.4	0.63	0.55	0.63	0.56	0.31	0.33
3.8	0.63	0.57	0.63	0.56	0.32	0.33
4.2	0.60	0.56	0.60	0.56	0.28	0.30
4.6	0.53	0.50	0.54	0.49	0.28	0.26
5.0	0.43	0.43	0.42	0.42	0.21	0.21

β-Glycerophosphates—(*a*) This was done by using taka-phosphatase and phthalate buffers without and with addition of Mg ions (results in Table III). The unknown appears to be identical with the authentic β-glycerophosphate. (*b*) Kidney phosphatase and

glycine buffers were used, without the addition of Mg ions (Table V). (c) Dialyzed kidney phosphatase and glycine buffers were used, with and without the addition of Mg ions (Table IV). In view of the definite differences in rate of hydrolysis of authentic α- and β-glycerophosphate by kidney and by taka-phosphatase already described it seems clear that glycerophosphate X (actually

TABLE IV

Comparison of Authentic and Unidentified Glycerophosphates, Using Dialyzed Kidney Phosphatase, with and without Mg

Hydrolysis results are given in mg. of P per 10 cc. of reaction mixture.

pH	Authentic β-glycerophosphate		Glycerophosphate X		Authentic α-glycerophosphate	
	Without Mg	With Mg	Without Mg	With Mg	Without Mg	With Mg
8.2	0.063	0.120	0.052	0.121	0.076	0.160
8.5	0.092	0.183	0.099	0.187	0.094	0.185
8.9	0.111	0.251	0.114	0.248	0.088	0.188
9.3	0.129	0.248	0.135	0.250	0.099	0.185
9.7	0.124	0.210	0.119	0.213	0.076	0.087
10.0	0.073	0.098	0.078	0.093	0.034	0.042

TABLE V

Comparison of Authentic β- and an Unknown Glycerophosphate, X, Using Kidney Phosphatase

Hydrolysis results are given in mg. of P per 10 cc. of reaction mixture.

pH	Authentic β-glycerophosphate duplicates		Glycerophosphate X duplicates	
8.5	0.089	0.090	0.091	0.091
8.9	0.162	0.162	0.165	0.163
9.3	0.169	0.170	0.165	0.167
9.7	0.140	0.139	0.142	0.139
10.0	0.098	0.098	0.101	0.101

Eastman Kodak "sodium glycerophosphate 644") must be mainly, if not completely, the salt of the β ester.

2. Comparison of the Enzymically Synthesized Ester with Authentic α- and β-Glycerophosphates—(a) Glycerophosphate was synthesized enzymically, with an intestinal phosphatase preparation, from sodium phosphate (Na_2HPO_4) and glycerol, by a method already described by one of us (7). The synthesized glycero-

phosphate, isolated as the barium salt, was transformed into the sodium salt, and the rate of hydrolysis by kidney phosphatase compared with that at which the pure authentic α- and β-glycerophosphates of the same concentration were hydrolyzed. (The results are shown in Table VI.) (b) After allowing another similar enzymic synthesis of glycerophosphate to proceed to equilibrium, the barium glycerophosphate separated from the reaction mixture was fractionally crystallized from water into three portions, the fraction least soluble in water (q), a fraction of intermediate solubility (r), the fraction most soluble in water (s).

Each of these portions was again recrystallized, and divided into three fractions in the same way. The least soluble (Q), a mixture

TABLE VI

Comparison of Enzymically Synthesized Glycerophosphate with Authentic α and β Esters, Using Kidney Phosphatase

Hydrolysis results are given in mg. of P per 10 cc. of reaction mixture.

pH	Authentic α-glycerophosphate	Authentic β-glycerophosphate	Enzymically synthesized glycerophosphate
7.8	0.110	0.124	0.108
8.2	0.133	0.144	0.130
8.5	0.177	0.196	0.172
8.9	0.203	0.235	0.198
9.3	0.196	0.241	0.198
9.7	0.127	0.208	0.130
10.0	0.067	0.122	0.076

of fractions of intermediate solubility (R), and the most soluble fraction (S) were separated.

King and Pyman (5) have reported that barium α-glycerophosphate is much less soluble than the barium salt of the β ester, and it was hoped that possibly fractions Q, R, and S would show some difference in their rate of enzymic hydrolysis.

The experimental findings are shown in Figs. 2 and 3, kidney phosphatase being used in the former and taka-phosphatase in the latter experiment. It is probable from these results that no large amount of β-glycerophosphate is produced, or rather remains in the reaction mixture at equilibrium, but that the glycerophosphate then present is mainly the α form. In this case it cannot, unfortunately, be stated outright that the synthesized product is mainly

the racemic α ester, since it is very possible, indeed likely, that the enzyme-synthesized product may be one or other of the optical α isomers, although neither the sodium nor the barium salts of the enzymically synthesized glycerophosphate, the former in 10 per cent, the latter in saturated aqueous solution, showed any detectable optical activity in a 2 dm. polarimeter tube. This however gives no information with regard to their derivation from d, l, or dl,α-glycerophosphoric acid, since Karrer and Benz (8) state that the barium and sodium salts of "optically active" α-glycerophosphoric acid are entirely without optical activity. Assuming for the present that the two optical isomers of α-glycerophosphate are hydrolyzed by the enzyme at rates not widely dissimilar from one another, we can make the estimate that, at equilibrium, the enzyme-synthesized glycerophosphate probably contains no more than 15 per cent of the β isomer.

There are at present two methods known for distinguishing between α- and β-glycerophosphoric acid, namely the color reaction of Denigès (9) which depends on the fact that α-glycerophosphoric acid, but not β-, gives on oxidation with bromine a derivative of dihydroxyacetone, which reacts with certain phenols in concentrated sulfuric acid to give intensely colored solutions, the color depending on the phenol used.

The other method is claimed to be more quantitative, and depends on the formation of a double salt, relatively insoluble in water, between barium nitrate and barium β-glycerophosphate (Karrer and Salomon (10)). Barium α-glycerophosphate does not appear to form such a double salt, and practically quantiative separation of a mixture of barium α- and β-glycerophosphate by this means is claimed.

We have confirmed the formation of a double salt between barium nitrate and barium β-glycerophosphate, and were unable to obtain such a salt either with authentic barium α-glycerophosphate or with the enzyme-synthesized barium glycerophosphate. This, as far as it goes, supports our findings shown in Figs. 2 and 3 that the enzyme-synthesized ester is mainly the α form. We find it impossible, however, to obtain such great solubilities for the barium salts of any of these glycerophosphates as the 10 per cent solubility required by Karrer and Salomon for the carrying out of their test, so that we are unable completely to con-

firm their findings with regard to the non-production of the double salt by the α isomer under the same conditions in which such a salt is formed by the β isomer. We have used, instead of 10 per cent solutions, saturated solutions of the three salts for this test. The solubility in gm. per 100 cc. solution of our authentic barium α-glycerophosphate is 1.7 at 24°, of our authentic barium β-glycerophosphate 5.1 at 25°, and of the three fractions of the barium salt of the enzyme-synthesized ester (Figs. 2 and 3) 1.6 gm. for Q, 2.1 gm. for R, and 2.3 gm. for S per 100 cc. at 17°. King and Pyman (5) found a solubility of 1.49 per 100 cc. at 13° for the α and of 6.7 gm. per 100 cc. at 12° for the β salt.

The method described in the present paper offers a third, and semiquantitative means for distinguishing between the two isomers. It is now being applied to the naturally occurring compounds of glycerophosphoric acid.

SUMMARY

A new method for identifying the dl,α and β isomers of glycerophosphoric acid is described. It depends on the fact that the two isomers are hydrolyzed at different rates by plant or animal phosphatases. Examples of the use of the method are given.

It would seem very advisable, in the description of experiments, particularly quantitative experiments, on the hydrolysis of glycerophosphate by enzymes, to specify whether the α or β form of the ester has been employed.

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CREATINE IN HUMAN MUSCLE

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Hunter (1) has tabulated the data obtained by various investigators with respect to the creatine content of normal human skeletal muscle. Chisolm (2), using Mellanby's method, analyzed specimens of muscle of six adults who had met sudden death and obtained a range of values of 251 to 290 mg., with an average of 270 mg. per 100 gm. of fresh muscle. This value he considered about 10 per cent below the actual creatine content, the difference being attributed to the loss of creatine from the tissues during the period (24 hours) the subjects were kept in the cold room before autopsy.

Two analyses of human muscle reported by Myers and Fine (3) give the following values for muscle creatine; abdominal muscle 0.396 per cent, muscle from the leg 0.391 per cent.

Shaffer (4) analyzed muscle obtained in three cases of sudden death and reported total creatine values ranging from 393 to 430 mg., with an average of 411 mg. per 100 gm. of muscle. Of this, the creatinine amounted to only 10 to 11.5 mg. per 100 gm. of muscle.

Denis (5) determined the creatine content of the psoas muscle, secured at autopsy in five cases of sudden death, and obtained values ranging from 360 to 421 mg., the average being 389 mg. per 100 gm. of muscle.

Cardiac muscle was found to contain 170 to 180 mg. per cent by Constabel (6).

The data of Myers and Fine, Shaffer, and Denis show good agreement, the average of all their analyses being 398 mg. per 100 gm. of muscle. As compared with the normal values recorded by these investigators, Bodansky, Schwab, and Brindley (7) obtained values ranging from 159 to 324 mg. per cent for the creatine content of different muscles in a case of generalized myositis

fibrosa. A relationship was apparent between the amount of creatine in the muscle and the degree of inflammation present. Thus, the iliacus, which histologically showed the greatest amount of inflammation, contained only 160 mg. of creatine, whereas the soleus, which showed the least inflammation, contained 324 mg. per 100 gm. of muscle. It was realized, however, that in the absence of normal creatine values for most of the muscles the data could not be evaluated with certainty. For example, the diaphragm muscle contained 159 mg. per cent of creatine, a value

TABLE I

Creatine (and Creatinine) Content of Human Muscle (Mg. per 100 Gm., as Creatine)

Muscle	Case 1, white		Case 2, negro		Case 3, negro	
	Rose, Helmer, and Chanutin method	Ochoa and Valdecasas method	Rose, Helmer, and Chanutin method	Ochoa and Valdecasas method	Rose, Helmer, and Chanutin method	Ochoa and Valdecasas method
Diaphragm................	331	357	309	364	309	364
Crus of diaphragm........					342	390
Myocardium.............	261	268	257	285	220	
Deltoid..................	383	421	451	476		
Sartorius................	449	442				
Temporal................			356	404	343	371
Psoas....................	485	476	465	483	448	476
Intercostal..............	290(?)	442			266 (?)	368
Pectoralis major.........	447	484	456	464	433	463
Supraspinatus...........	396	432				
Soleus..................	398	422				

which was assumed to be low, although there were no normal analyses in the literature with which to compare the result.

Since then analyses have been made of various muscles obtained in three cases of sudden death due to accident. All were males, between the ages of 25 and 32 years. The results of these analyses, outlined in Table I, fully justify the assumptions which were made by Bodansky, Schwab, and Brindley (7).

In the analyses recorded in the paper to which reference has been made (7), the method of Ochoa and Valdecasas (8) was employed. This procedure was originally adopted with the intention of using

it in the analysis of muscle obtained at biopsy where the amount of material available to us would have been too small for other analytical procedures, but sufficient to carry out the Ochoa-Valdecasas analysis in triplicate, 100 mg. of muscle being used for each determination. In the analyses recorded in the present paper, both this method and that of Rose, Helmer, and Chanutin (9) have been employed. Where sufficient material is available and the muscle is fairly uniform in texture, Rose's method presents certain advantages, the results being more uniform than those obtained by the method of Ochoa and Valdecasas. On the other hand, with muscle containing large amounts of connective tissue, such as the intercostal muscles and diaphragm, more satisfactory results may be obtained by the Ochoa-Valdecasas procedure, since it is relatively easy to tease out, with fine forceps, individual muscle strands in sufficient amount for this determination.

SUMMARY

The methods of Rose, Helmer, and Chanutin and of Ochoa and Valdecasas have been used in the analysis of various muscles for their creatine content. The values obtained ranged from 220 mg. per cent for cardiac muscle to 485 mg. per cent for the psoas muscle.

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STUDIES IN GASTRIC SECRETION

I. GASTRIC JUICE OF CONSTANT ACIDITY*

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The question as to whether the concentration of hydrochloric acid in the freshly secreted gastric juice is constant or not continues to evoke much debate. So extensive have been the contributions already made to this field of endeavor that the value of any new efforts directed toward securing data conclusive with respect to this problem is obvious. Furthermore, it might be expected that a settlement of this question would in itself constitute an important contribution to our knowledge of the mechanism of formation of the gastric hydrochloric acid. For these reasons the experiments reported in this paper were planned and carried out.

HISTORICAL

It is unnecessary to review here the extensive literature bearing on the question. In view of the pronouncedly diverse opinions that have been expressed concerning this problem, however, and in order to orient the reader quickly, it seems worthwhile to present a brief résumé of the kind of evidence that has been cited for and against the idea that the concentration of hydrochloric acid in the freshly elaborated gastric juice is constant.

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Evidence in Support of Constancy of Gastric Acidity

In 1879, Heidenhain reported approximate constancy in the concentration of gastric acid in several series of gastric samples collected from dogs. Subsequently (1883) he suggested the possibility that such constancy may be characteristic of the secretion as it is initially synthesized by the glands. No significant contributions to the problem were made thereafter until Pavlov began his investigations and created the starting point of the present controversy. Pavlov (1910) has claimed "that the gastric juice as it flows from the glands possesses a constant acidity" but this frequently quoted statement is only a hypothesis and not a direct inference from his experimental observations. In support of this we submit the following context:

"All the more astonishing, therefore, . . . appears the fact, *as it seems to be,* [italics ours] that the gastric juice as it flows from the glands possesses a constant acidity. . . . *We do not, however, receive the juice directly from the glands,* even in our method. After it is secreted by these it has to flow over alkaline mucous membrane and inevitably becomes more or less neutralized, that is to say, has its acidity reduced. To this circumstance must be attributed the apparent fluctuations of acidity, as is clearly shown by numerous observations. *It is a rule almost without exception that the acidity of the juice is closely dependent upon the rate of secretion;* the more rapid the latter, the more acid the juice, and vice versa. . . . Obviously this is because the stream of juice has been neutralized by the mucus, and if the stomach has been washed so to speak, in this manner several times in succession, not unfrequently all connection between rate of secretion and degree of acidity can be removed. That is to say, the juice is equally and strongly acid whether it be rapidly or slowly poured out. . . . Additional support is given to the *theory of constant acidity* by the following. . . . [pp. 32–34]. The variations in acidity are often considerable, but we have no reliable proof to show that they occur in the juice as it flows from the gastric glands. . . . Nevertheless the possibility of a more intimate relationship between acidity and rate of flow is not wholly excluded, such, for instance, as exists in the case of the salts of saliva" (p. 124).

From the last sentence, it is apparent that Pavlov himself was not entirely convinced of the validity of this generalization, particularly when data obtained from different individuals and in response to different stimuli are considered.

Evidence in favor of Pavlov's view has been advanced by various investigators from studies of (*a*) experimental gastric pouch animals with particular reference to the parallelism of acidity with secretory rate, (*b*) gastric fistula cases in human beings, and (*c*) various types of clinical cases examined by the fractional analysis technique.

Evidence from Studies with Experimental Animals—Among workers with animals, Pavlov's staunchest supporter has been Boldyreff (1915), who, however, gave no evidence of his own in support of the constancy of the maximum value for the concentration of gastric acid, but cited a number of

other investigators instead, *e.g.* Umber (1905), Pfaundler (1900), Hornborg (1904), Goorevich (?), Moritz (1901), Seiler (1901), Verhaegen (1896), and Sommerfeld (1905). More recently, Lim and Liu (1926) attempted to fatigue the gland cells of Pavlov pouch dogs by repeated injections of histamine, and concluded from their observations that HCl formation follows the "all or none" law; *i.e.*, the concentration of hydrochloric acid is constant and independent of intensity of stimulation; the volume of acid juice secreted per unit time, however, is a function of the extent of stimulation. Likewise, McCann (1929) observed the common parallelism of acidity and secretory rate in dogs, using the fractional technique for gastric analysis. Changes in total acidity were explained solely on the basis of the Heidenhain-Pavlov theory; the possibility of a variation in the concentration of HCl as it was first synthesized was entirely discarded.

In their work with stomach pouches in cats, Gamble and McIver (1928) found marked constancy in the total ionic content of the juice. Even though the fixed base varied widely, and therefore also the acidity (which was determined by difference of total chloride and fixed base values), these workers inferred the constancy of the original acid secretion from that of ionic content. The variations observed in the calculated acid values were ascribed in the usual way to admixture of a mucous secretion.

Evidence from Clinical Material—From numerous sham feeding experiments on a human fistula case, Kaznelson (1907) concluded that the gastric acid is secreted at a constant maximum concentration. Her data, however, are far from being conclusive; in one series, for instance (Table I of her paper), the values showed a random variation from 0.14 N (0.51 per cent, pH 0.94)[1] to less than 0.12 N (0.44 per cent, pH 1.00). Subsequently, Rehfuss and Hawk (1914) reported what they considered to be "the first direct demonstration in the human subject in favor of Pawlow's theory of the secretion of a juice of constant acid concentration." Their experiments consisted of a series of fractional analyses on both normal and pathological individuals. Not only is there a lack of really good agreement among the values found for different individuals, but the variation shown in the region of the maximum for any one subject proves to be as high as fifteen clinical units. Furthermore, the greatest of these values is hardly comparable with that found by Kaznelson in her human fistula case.

On the basis of an analysis of their clinical records Bloomfield and Keefer (1926) concluded that "under uniform conditions of examination a normal person secretes a gastric juice of *highly constant acidity* . . . The vol-

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[1] There is no uniformity in the terminology employed by the workers in this field to represent acid concentration; per cent, pH, normality, and "clinical units" have all been used according to the individual preference of the investigator. Consequently, in this paper, whenever an acid value is quoted in per cent, its equivalents will be inserted parenthetically. Inasmuch as clinical units and millimolar concentration are numerically identical, and equal to 1000 times the normality, only the latter and the pH values will be employed.

ume of secretion and the degree of acidity vary within considerable limits in different people but under the conditions of these experiments are practically constant in the same person on repeated examinations."

If it be true that the concentration of the acid as it is secreted by the parietal cells may vary with the conditions obtaining at the time of secretion, one might expect such a variation to be even more marked in clinical cases of gastric hypersecretion. Reference to so extensive a compilation as that of Rehfuss (1927) indicates the general acceptance by clinical investigators of the view that a true hyperacidity in the above sense does not exist. One of us (Hollander, 1930) has recently reported very direct evidence on this point. In a gastric pouch dog there was observed a case of hypersecretion in which the average rate of flow was increased more than 10-fold. Nevertheless, the acidity remained constant within very narrow limits.

Evidence in Refutation of Constant Gastric Acidity

Two lines of investigation have been most productive of evidence cited in opposition to Pavlov's theory. The first of these is based on comparisons of gastric acidity with rates of secretion leading to the observation that acidity decreases during the latter part of the digestive period, after having previously risen to a high value. In consequence of such observations, Rosemann (1907) objected to Pavlov's idea on the ground that all of the mucus would have been washed out during the early part of the experiment thus eliminating the possibility of further neutralization. This point of view was supported by the studies of Foster and Lambert (1908, *a*, 1908, *b*), King and Hanford (1918), and Carlson (1923). As for the second of these lines of attack, Rosemann called attention to the fact that the total chlorine content of the gastric juice is very nearly constant. This led him as well as other later investigators (MacLean and Griffiths, 1928; Close, 1929; Webster, 1929) to favor the view that the chloride ion brought to the gland is secreted at a definite fixed concentration, part of it unchanged as sodium chloride and part converted into hydrochloric acid, and that the extent of this change governs the acidity of the secreted juice.

. It is doubtful whether the studies made on so called "pure" gastric juice obtained, for example, by the use of a stomach tube

for the collection of gastric juice (Garbat, 1925, and Delhougne, 1926) really contribute much of value to a solution of the question under consideration. Much higher values for total acidity have been reported by so many other workers that the purity of the secretions obtained by these techniques may well be questioned.

Discussion and Summary of Evidence Just Cited from the Literature

None of the evidence cited above is conclusive with respect to the validity of either Pavlov's theory or Rosemann's hypothesis. The proponents of both views seem to be in accord with respect to the following facts: (1) except in isolated instances, appreciable variations in total acidity always occur; (2) combined acidity, determined as the difference between free and total, is always significantly large with its magnitude in general increasing as the total acidity diminishes; (3) total chloride is relatively constant in pouch juice, whereas, the values fluctuate over a very large interval in the case of stomach contents; (4) neutral chloride, determined directly as total base or as the difference between chloride and acidity, always increases as the acidity decreases and conversely.

It is conceivable that these variations may result from either of two mechanisms, if not both acting simultaneously. First, in accord with the suggestion of Pavlov, there may occur an admixture of one or more "foreign" substances with a hydrochloric acid secretion of uniform concentration. If the foreign substance contains alkali and alkaline earth bicarbonates in relatively small though appreciable quantity, and chlorides in much greater proportion, with traces of phosphate, protein, etc.—as do occur in the mucous and other alkaline secretions—then all of the fluctuations enumerated above can be accounted for merely on the basis of variations in the relative amounts of HCl and the "contaminating" substance. On the other hand, it is conceivable that such fluctuations are inherent in the original acid secretion; in other words, that the various alkali and alkaline earth salts come in greater part from the parietal cells.

It is the purpose of this paper to report a series of experiments with gastric pouch dogs in which the relations of the acidity of gastric juice to rate of flow of the secretion, method of collection

of the samples of juice, and admixture with mucus were studied. Two methods were found by which gastric juice of relatively constant pH may be obtained from pouch dogs. The value of this "normal" constant acidity was found to be independent of the individual animal, of the stimulus employed, and of the rate of secretion.

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EXPERIMENTAL

Preparation of Gastric Pouch Dogs

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The data presented here were obtained from five mongrel dogs (four females and one male) provided with accessory stomach pouches.   Dogs E, G, J, and O had fundic pouches of the Pavlov type; Dog Q had a Heidenhain pouch.   In the case of Dog J, the first operation provided a Pavlov pouch.   Later, after a perforation of the septum occurred, a repair operation was performed which changed the accessory stomach to that of the Heidenhain variety.

Because of its importance in relation to the problem under investigation, it is advisable to give some details concerning our operative procedure.   In our earlier operations (Dogs E and G), the pouch was delivered through a left rectus stab wound and anchored to the abdominal wall in the usual way.   Many months after the operation, fairly good retention of fluid was observed in both cases, but only after skin erosion had been controlled sufficiently to permit of the formation of scar tissue around the mouths of the pouches.   Such a retention of juice within the pouch has already been reported by others (Dragstedt, 1917; Gamble and McIver, 1928).

Later in our work, however, it became apparent that development of such a sphincter action at the mouth of the pouch at the earliest possible time after the operation would be of great value in this research.   This was finally accomplished by bringing the pouch out through an opening in the oblique muscles made by carefully separating the fibers in each layer by blunt dissection. The pouch was anchored as before.   In 2 to 3 weeks this sphincter was found to be so effective as to retain fluid under considerable pressure for several hours.   However, when secretion continues under these conditions, it has been noted that the pressure may ultimately become so great as to force the sphincter with loss of contents into the dressing.

Care of Animals

Because the exudate from an eroded area surrounding the opening of the pouch has appreciable buffering power and therefore may reduce the acidity of the collected gastric juice samples unless special precautions are taken, we wish to record here our experience in combatting the skin erosion that results from continual leakage of the gastric juice. That this experience is common to most investigations in this field is suggested by the numerous records of attempts to deal with it. For our work retention devices such as Carlson, Orr, and Brinkman (1914) and Boldyreff (1925) used were undesirable because a foreign body is known to increase the flow of mucus and perhaps also of an exudate—particularly if it be sutured in place; continuous collection of the juice every day was impossible because of lack of manual assistance to care for all the animals used. Consequently a number of chemical dressings to neutralize the acid were tried. Petrolatum, zinc ointment, calcium carbonate, magnesium carbonate, and magnesium oxide all failed to prevent the formation of large areas about the mouth of the pouch where muscle as well as skin was digested away. The situation was finally controlled in the following manner. The entire area was covered with precipitated chalk over which was placed a piece of gauze thinly covered with zinc ointment. This in turn was held in place by a thick pad of cellu cotton, the whole being covered with a jacket laced on the left side and provided with holes for the forelegs. The lacing with string made dressing the animals a very simple and rapid affair. Ordinarily the dressing was changed twice a day. By this procedure it has been found possible not only to control the erosion but actually, as in the case of Dog G, completely to heal a large area which had been eroded for more than a year. The sphincter action described above is also of great value in reducing this autodigestion, especially when combined with frequent catheterization of the pouch; but if the retained juice is permitted to flow out into the dressing, only the above combination of chalk and zinc ointment has been found to be satisfactory in minimizing its erosive effects.

All of the dogs were housed in metabolism cages. The usual food was a modification of the Karr-Cowgill casein Diet 2 (Cowgill, 1921) with a 50 per cent increase in sodium chloride content

to compensate for chloride lost by way of the gastric juice. This food mixture is adequate for canine nutrition when suitably supplemented with sources of vitamins. Twice a week the animals received liberal amounts of vitavose[2] as a source of the vitamin B complex and cod liver oil as a carrier of the fat-soluble vitamins. In order to introduce some variety as well as to reduce the expense of feeding the dogs the routine diet was occasionally replaced by cooked or raw meat, meat residue,[3] or commercial dog biscuit and milk powder.

Technique for Collection of Gastric Juice

Inasmuch as the present investigation centers about the influence of mucus secretion on the acidity, it was obvious from the outset that the juice must be collected, if possible, by some device which will not augment the mucus flow by mechanical irritation of the membrane of the pouch. Furthermore, the device must be such that exudate from the eroded skin, no matter how slight, will be entirely excluded from the fluid collected. In those experiments which made use of the pouch sphincter (here called the *discontinuous collection experiments*) the solution was a simple one. The dog was left in its cage as usual. At such times as it was desired to collect the accumulated fluid the dressing was removed, the mouth of the pouch as well as the surrounding area carefully cleaned, and a small soft rubber catheter (sizes 12 to 16, French) inserted for just long enough to draw off the juice. Usually, slight pressure on the abdominal wall over the pouch was sufficient to insure adequate evacuation. Several times the fluid was sucked out with a syringe, but almost invariably the mucosa was drawn against the eye of the catheter with some traumatization. Therefore this procedure was not adopted as a routine.

For the *continuous collection experiments*, however, when it was desired to collect the secretion by continuous drainage—the dog being supported in an upright position by means of a sling—a

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[2] A commercial preparation of the vitamin B complex obtained from E. R. Squibb and Sons, New York.

[3] The material remaining after removal of extractives in the preparation of commercial beef extract. Obtained from the Valentine Meat Juice Company, Richmond, Virginia.

mechanical contrivance of some sort is essential.   Pavlov (1902)
used a simple multiple-eyed rubber catheter with a narrow flange
just beyond the last eye.   Of all the devices described in the litera-
ture this seemed to give promise of affording the minimum of irrita-
tion of the gastric mucosa.   The arrangement finally perfected for
use in the *continuous collection experiments* reported in this paper
was designed on much the same principles.

The completely assembled apparatus is shown in Fig. 1.   *A*
is a soft rubber catheter (sizes 14 to 16, French) with solid tip and
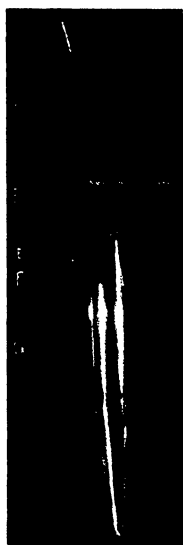
Fig. 1. Device for collecting gastric juice

three or four pairs of eyes punched in it.   The catheter is draw
through a small hole in the flange *B* in such a way that the lowest
eye is 20 to 25 mm. above it.   The flange is a 4 to 5 mm. disc
cut from a No. 12 or 14 solid rubber stopper.   By means of this
device any serous exudate as well as any gastric juice which may
have leaked down the outside of the catheter will be kept out of
the collected sample.   The flange is held firmly against the abdom-
inal wall by a wide band of canvas which is perforated for the
passage of the catheter and the brass screws *C*.   The ends of the
band are fastened over the dog's back by a hemostat or similar

instrument. The receiving vessel is a 15 cc. graduated centrifuge tube G passing through the center of another rubber disc D.   This second disc is held in place by the screws F, with two pairs of nuts, C and E, holding both the screws and the disc firmly in place.   If several such disc and tube combinations are always at hand, the collection vessel can be changed at any instant during the experiment without the loss of a single drop of fluid.   Also, no matter how much the dog may move, particularly when it becomes restless during a long experiment, there is no possibility of losing the sample, as exists with other contrivances.   The arrangements for supporting the animal during an experiment were essentially those illustrated by Boldyreff (1925).

The procedure in all these *continuous collection experiments* was fairly uniform.   The animal was suspended in the sling and the collecting device attached.   At least 1 hour thereafter (or long enough to insure the absence of any marked acid reaction from the previous meal) the animal was fed the indicated meal or given the injection of histamine.   The minute at which food was offered was taken as zero time.   At convenient intervals thereafter, usually 15 or 30 minutes, depending on the rapidity of secretion, the collecting tube was changed.   In the tables, the column headed "time" gives the number of minutes after zero time when collection of that particular sample was discontinued.   Volumes were read directly from the graduations on the tube.   Histamine was always given subcutaneously in the region of the left scapula.   No regularity was attempted with respect to the amount of food ingested or in the volume of fluid injected, although this latter volume never exceeded 3 cc.   In general, collection of juice was continued until the secretory rate reached a recognizably low value.   For purposes of comparison, the "rate of secretion" values in the tables have all been calculated as cc. per 15 minute intervals.   In plotting pH as ordinates, a descending scale has been used, corresponding to a scale of increasing acidity.

Determination of Acidities

Because of the great number of samples to be examined and also because there was frequently only a very small volume of juice available, acidities were measured electrometrically instead of titrimetrically.   The relative advantages and disadvantages of

these two methods, together with all manipulative details, will be described in the second paper of this series. The determinations were made with a quinhydrone electrode designed to use no more than 0.2 cc. of liquid for a measurement. Since equilibrium was usually attained in 3 to 5 minutes, the E.M.F. remaining constant for at least 30 minutes, it was possible to examine a large number of gastric juice samples simultaneously. Any steady drift in the voltage, such as was occasionally observed, was sufficient grounds for discarding the determination. All samples were done in duplicate. As an indication of the accuracy attained in this work, the following may be cited.

Sixteen determinations of E.M.F. carried out on a single large sample of gastric juice gave an average deviation (a.d.) of ± 0.0004 volts. Similarly, 66 determinations with a standardized 0.1 N solution of HCl carried out over a period of about 6 months, gave an average of 0.3902 volts (a.d. ± 0.0003 volts). In view of the fact that 0.1 pH is equivalent to 6 millivolts, it may be assumed that all values cited here are subject to a variation of $\pm$ 0.01 pH.

All samples were filtered or centrifuged and decanted after collection to free them of mucin clots. Any suggestion of the presence of blood, no matter how faint, necessitated discarding the sample of juice which contained it. This was necessary for two reasons: (1) to eliminate any low acidity values which might result from neutralization by blood buffers; (2) the presence of hemoglobin invariably caused a drift in E.M.F. thus rendering it impossible to make a satisfactory measurement of pH. Whenever pH determinations could not be made immediately after collection of the secretion, the tubes were tightly stoppered and kept in a refrigerator. Preliminary studies showed that no significant change in acidity is induced in such tubes by filtration, centrifuging, or standing for 3 or 4 weeks under the above conditions. Evaporation resulting from ineffectual stoppering will bring about a decrease in pH, due to increased concentration of the liquid. Also, the progressive solution of unfiltered mucin will decrease the free acidity and therefore increase the pH, although the total acidity may not be affected significantly.

Observations

Series I. Continuous Collection Series with Food as Stimulus

In the first series of experiments (Table I) an attempt was made merely to repeat Pavlov's early observations on postprandial

TABLE I

Series I. Continuous Collection Experiments with Food as Stimulus

	Sample No.	Time	Volume	Secretion rate	pH (± 0.01)
		min.	*cc.*	*cc. per 15 min.*	
Experiment B 4. Dog G; stimulus, Karr-Cowgill casein diet and milk	B 4.1	30	1.6	0.8	3.74
	B 4.2	60			
	B 4.3	90	2.7	1.4	1.61
	B 4.4	120	2.4	1.2	1.49
	B 4.5	150*	1.8	0.9	3.14
	B 4.6	180	2.4	1.2	1.65
	B 4.7	210	2.6	1.3	1.59
	B 4.8	240	3.3	1.7	1.31
	B 4.9	270	2.5	1.3	1.48
	B 4.10	300	2.4	1.2	1.50
	B 4.11	330	2.1	1.1	1.80
Experiment B 19. Dog O; stimulus, Karr-Cowgill casein diet and beef extract	B 19.1	30	0.9	0.5	1.51
	B 19.2	60	1.4	0.7	1.07
	B 19.3	90	1.1	0.6	1.02
	B 19.4	120	1.1	0.6	1.01
	B 19.5	150	1.1	0.6	1.01
	B 19.6	180	1.5	0.8	1.02
	B 19.7	210	1.2	0.6	1.02
	B 19.8	240	1.1	0.6	1.05
	B 19.9	270	1.4	0.7	1.09
Experiment B 23. Dog J; stimulus, Karr-Cowgill casein diet and beef extract	B 23.1	30	0.6	0.30	
	B 23.2	90	0.6	0.15	1.49
	B 23.3	150	0.6	0.15	1.60
	B 23.4	210	0.8	0.20	1.51
	B 23.5	270	0.8	0.20	1.29
	B 23.6	330	0.8	. 0.20	1.25

* Small amounts of food were given near the end of this period.

variations in acidity. Consequently, these experiments were all performed by the continuous collection technique as described above. As can be seen from Table I and the illustrative curves
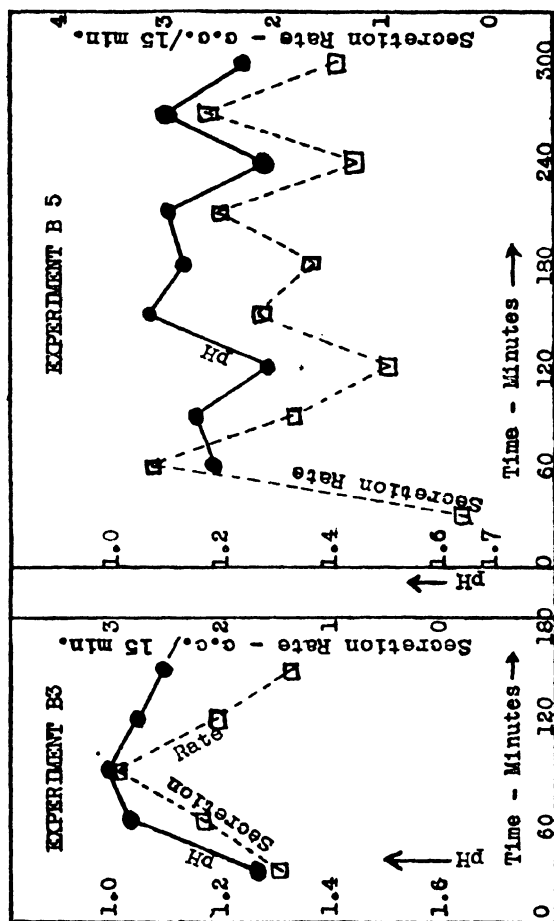
FIG. 2. Continuous collection experiments with food as stimulus. Experiment B 3 was performed on Dog O with meat residue as stimulus; Experiment B 5, on Dog G on the Karr-Cowgill casein diet and milk.

(Fig. 2), the parallelism between acidity and volume is confirmed beyond any possibility of doubt.   In Experiment B 3, the secretory curve was a simple one; both it and the acidity curve rose to a maximum and fell off immediately thereafter.   In Experiment B 4 a similar curve was obtained.   However, a small amount of food eaten toward the end of the period resulted in a second rise in the curve.   Here again the parallelism persisted as long as observations were continued.   Experiment B 5 is of particular interest. For some unknown reason, the secretory rate varied irregularly throughout the experiment.   In spite of these fluctuations, the relation between the two variables continued throughout.   In one place in his book Pavlov (1910) remarked that occasionally he had noted that the acidity maintained a constant value for an appreciable length of time.   Experiment B19 shows this same behavior.   The effect here, however, is probably fortuitous and arises only because of the absence of marked variation in the rate curve.

All four of these experiments were performed with Pavlov pouch dogs.   Experiment B 23 was made with Dog J at a time when the postprandial secretory rate was exceedingly low.   Subsequently, the response to food disappeared almost entirely, although the response of the pouch to histamine remained high.   Nevertheless, even this experiment gives manifest evidence of the correlation between acidity and rate of secretion.

Series II.   Continuous Collection Series with Histamine as Stimulus of Low Secretory Rate

The second series includes Experiments B 16, B 18, B 20, and B 26, the data for which are presented in Table II and Fig. 3.   In this set, food was replaced by histamine as a gastric stimulant, in order to determine whether the close parallelism between acidity and secretory rate is equally observable with the latter substance. Inasmuch as the highest rate attained in these experiments is 3.7 cc. per quarter hour (Sample B 16.4) while the greatest postprandial value recorded here is 3.2 (Sample B 5.2), these two sets of observations are comparable with respect to secretion rate.   ·

None of the records of these experiments shows the zig-zag effect occasionally observable in the previous ones.   Invariably the rate curve rises to a maximum value as illustrated in Fig. 3,

Experiment B 20, and drops off again directly.   In all cases there is a parallel increase in acidity, attaining its highest value simultaneously with the velocity maximum.   It may be concluded, therefore, that the histamine curves demonstrate the same rela-

TABLE II

Series II.   Continuous Collection Experiments with Histamine as Stimulus of Low Secretory Rate

	Sample No.	Time	Volume	Secretion rate	pH (± 0.01)
		min.	*cc.*	*cc. per 15 min.*	
Experiment B 16.* Dog	B 16.1	15	2.3	2.3	1.06
O; stimulus, 0.2 mg.	B 16.2	30	2.6	2.6	0.98
histamine per kilo	B 16.3	45	2.8	2.8	0.95
	B 16.4	60	3.7	3.7	0.94
	B 16.5	75	3.7	3.7	0.93
	B 16.6	90	2.9	2.9	0.95
	B 16.7	105	2.6	2.6	0.96
	B 16.8	120	1.9	1.9	1.00
	B 16.9	135	1.1	1.1	1.12
	B 16.10	150	0.9	0.9	1.22
Experiment B 18. Dog	B 18.1	30	2.1	1.1	1.13
O; stimulus, 0.2 mg.	B 18.2	45	1.8	1.8	0.95
histamine per kilo	B 18.3	60	1.4	1.4	0.94
	B 18.4	75	0.4	0.4	1.02
	B 18.5	135	0.5	0.1	
Experiment B 26.† Dog	B 26.1	30	2.5	1.3	1.03
O; stimulus, 0.4 mg.	B 26.2	48	1.7	1.4	0.94
histamine per kilo	B 26.3	82	3.2	1.4	0.95
	B 26.4	102	1.1	0.9	0.98

* All samples were slightly opalescent.

† Dog vomited shortly before the injection of histamine, probably because it licked some zinc ointment just before the experiment.

tion between acidity and rate as was observed when food was taken.

Series III.   Continuous Collection Series with Histamine as Stimulus of High Secretory Rate

Inasmuch as the acidity varies at least qualitatively with the rate of secretion, up to a rate of 3 to 4 cc. per quarter hour, the effect of a
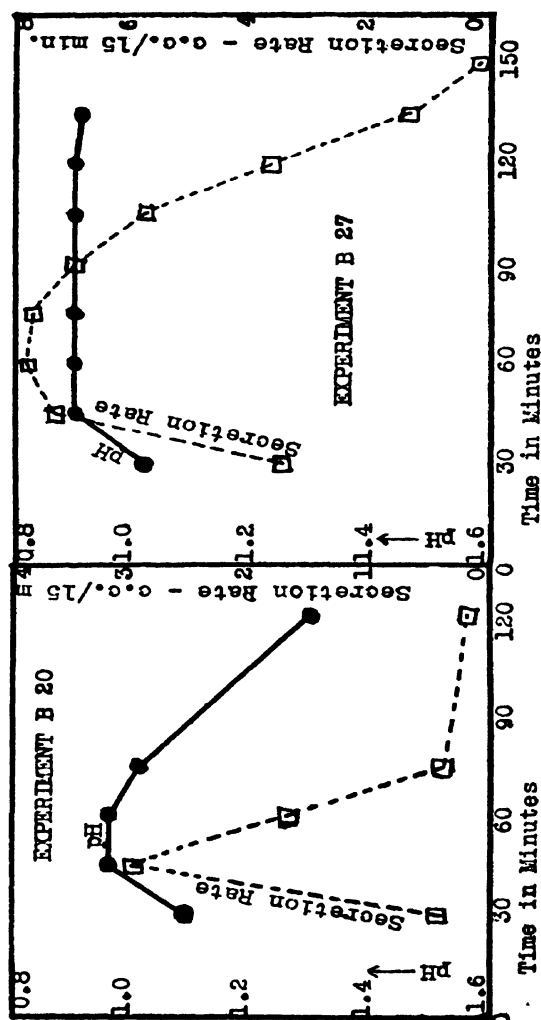
Fig. 3. Continuous collection experiments with histamine as stimulus. Experiment B 20 was performed on Dog J with a low secretory rate stimulated by 0.05 mg. of histamine per kilo; Experiment B 27, on Dog Q with a high secretory rate stimulated by 0.4 mg. of histamine per kilo.

further increase in this velocity is a matter of much importance. One might find that no matter how much the rate of secretion be augmented, its parallelism with the acidity persists. On the other hand, a point might be reached beyond which the acidity no longer shows any significant variation. Accordingly, a third series of experiments (Table III) was performed, differing from the previous series only in the relative rates of secretion. In general 0.4 mg. of histamine per kilo of body weight increased the rate sufficiently for the purpose. By this means it was possible to obtain a value as high as 7.8 cc. per quarter hour (Sample B 27.3). It must be remembered, however, that the relation between dose and magnitude of response is by no means the same for all dogs.

The experiments cited in Table III show that under these conditions the correlation between the two variables disappears entirely (see Fig. 3, Experiment B 27). In most cases the rate curve has the same general shape as have those in the previous experiments. In two instances (Experiments B 13 and B 17) a second peak exists due to secondary disturbances of unknown origin. Notwithstanding this conformity in the rate curves, the pH curves all rise to a maximum acidity (or minimum pH value) after one or two samples and stay there until the rate of secretion has fallen off considerably. Sometimes the acidity curve attains its maximum value even before the velocity curve does, as in Experiments B 15 and B 27. With but one exception— Experiment B 17—the acidity never departs from the maximum until the rate has fallen to 1.0 cc. or lower. In this instance the change in slope of the pH curve is first manifested at a secretory rate of 1.6 cc. (Sample B 17.8). Such constancy, it may be noted, is as good as the method of measurement permits; i.e., ± 0.01 pH. And it persists even in those experiments which show a double peak.

Let us consider the magnitude of this maximum acidity, or minimum pH. In the eight experiments cited, the pH values of the plateaus are as follows: Experiment B 13, 0.91; Experiment B 14, 0.91; Experiment B 15, 0.91; Experiment B 17, 0.93; Experiment B 21, 0.91; Experiment B 27, 0.90; Experiment B 28, 0.90; Experiment B 29, 0.90 (all ±0.01). These limiting pH values show a truly remarkable degree of uniformity (0.91 ± 0.02, equivalent to a free acidity of 0.15 N).

TABLE III

Series III. Continuous Collection Experiments with Histamine as Stimulus of High Secretory Rate

	Sample No.	Time	Volume	Secretion rate	pH (± 0.01)
		min.	*cc.*	*cc. per 15 min.*	
Experiment B 13.* Dog	B 13.1	30	4.7	2.4	
J; histamine dose, 0.2	B 13.2	45	5.5	5.5	0.91
mg. per kilo	B 13.3	60	5.4	5.4	0.91
	B 13.4	75	5.4	5.4	0.91
	B 13.5	90	2.6	2.6	0.92
	B 13.6	105	3.2	3.2	0.92
	B 13.7	120	1.0	1.0	0.95
	B 13.8	165	0.6	0.2	1.11
Experiment B 14. Dog	B 14.1	30	4.3	2.2	1.14
G; histamine dose, 0.2	B 14.2	45	3.4	3.4	0.91
mg. per kilo	B 14.3	60	2.9	2.9	0.91
	B 14.4	75	2.2	2.2	0.91
	B 14.5	90	2.1	2.1	0.92
	B 14.6	120	0.7	0.4	0.99
Experiment B 15. Dog	B 15.1	30	0.8	0.4	1.29
J; histamine dose, 0.2	B 15.2	45	2.9	2.9	0.97
mg. per kilo	B 15.3	60	3.7	3.7	0.92
	B 15.4	75	5.5	5.5	0.92
	B 15.5	90	3.3	3.3	0.91
	B 15.6	105	2.1	2.1	0.91
	B 15.7	120	0.6	0.6	0.94
	B 15.8	165	0.4	0.1	
Experiment B 17. Dog	B 17.1	15	2.0	2.0	1.48
G; histamine dose, 0.4	B 17.2	30	3.9	3.9	0.96
mg. per kilo	B 17.3	45†	4.4	4.4	0.93
	B 17.4	60	3.3	3.3	0.93
	B 17.5	75	3.6	3.6	0.93
	B 17.6	90†	2.7	2.7	0.93
	B 17.7	105	1.9	1.9	0.94
	B 17.8	120	1.6	1.6	0.98
	B 17.9	135	0.8	0.8	0.99
	B 17.10	165	1.1	0.6	1.11
	B 17.11	195	0.7	0.4	1.36
Experiment B 21. Dog	B 21.1	30	2.2	1.1	1.19
O; histamine dose, 0.4	B 21.2	45	2.3	2.3	0.94
mg. per kilo	B 21.3	60	2.0	2.0	0.92
	B 21.4	75	1.5	1.5	0.92
	B 21.5	90	0.5	0.5	0.91

TABLE III—*Concluded*

	Sample No.	Time	Volume	Secretion rate	pH (± 0.01)
		min.	*cc.*	*cc. per 15 min.*	
Experiment B 28. Dog	B 28.1	15	1.6	1.6	1.29
Q; histamine dose, 0.4	B 28.2	30	6.2	6.2	0.95
mg. per kilo	B 28.3	45	6.3	6.3	0.90
	B 28.4	60	6.2	6.2	0.90
	B 28.5	75	5.9	5.9	0.90
	B 28.6	90	2.9	2.9	0.91
	B 28.7	105	0.3	0.3	0.92
Experiment B 29. Dog	B 29.1	15	1.8	1.8	1.25
Q; histamine dose, 0.4	B 29.2	30	5.5	5.5	
mg. per kilo	B 29.3	45	6.2	6.2	0.90
	B 29.4	60	5.8	5.8	0.89
	B 29.5	75	4.5	4.5	0.89
	B 29.6	90	3.9	3.9	0.90
	B 29.7	105	1.4	1.4	0.91
	B 29.8	120	0.4	0.4	

* Histamine was injected 2.5 hours after feeding.

† Dog vomited during each of these periods. Shortly after this experiment the animal became sick with a gastrointestinal disturbance. Even on this day, in fact, loss of appetite was already observable.

Series IV. Discontinuous Collection Series, with Food as Stimulus

One of our first successful pouch operations was carried out with Dog E. For a long time the animal was not used for experimentation, but one day it was observed that the mucosa had retracted and scar tissue had formed sufficiently to retain large volumes of juice for a number of hours. Unfortunately, only a few such samples of retained juice were collected for examination before the skin closed over the mucosa entirely; these samples are included in the C 1 experiments in Table IV. In carrying out these experiments only the usual diets were employed as stimuli, the food having been given the dog in the morning after the pouch was emptied of whatever secretion it may have retained from the previous day. The average of these pH values is 0.92, in exact agreement with the values later obtained by the continuous collection technique from the other four dogs. The concordance of

TABLE IV

Series IV. Discontinuous Collection Experiments with Food as Stimulus

	Sample No.	Time	Volume	pH (⊷ 0.01)
		hrs.	*cc.*	
Experiment C 1 (pre-	C 1.1	6		0.93
liminary*). Dog E	C 1.2	7	12	0.93
	C 1.4	6½	26	0.90
Experiment C 2 (pre-	C 2.1	7¼	5.0	0.91
liminary†). Dog G	C 2.2	9	4.5	0.92
	C 2.3	7	1.3	1.03
	C 2.5	7	1.1	1.06
Experiment C 2.‡	C 2.6	7½	10.0	0.91
Dog G	C 2.7	8	13.0	0.91
	C 2.8	4½	1.4	0.93
	C 2.9	6	2.0	0.97
	C 2.10	7½	5.0	0.90
	C 2.11	4½	8.0	0.91
	C 2.12	5¼		0.91
	C 2.13	5½	4.0	0.91
	C 2.14	3¼	0.4	1.06
	C 2.15	4	1.0	0.96
	C 2.21		10.0	0.91
Experiments C 4 and	C 4.6	9	2	0.91
C 8. Dog O	C 4.7	6	5	0.98
	C 4.8	5¼	18	0.92
	C 4.9	5	15	0.94
	C 4.10	5	6	0.97
	C 4.11	5	12	0.94
	C 4.12	4	18	0.92
	C 4.14	4¼	5	0.91
	C 4.15	3	3	1.55
	C 4.16	2¾	3	1.05
	C 4.20	3	13	0.94
	C 4.22	4	20	0.93
	C 4.23	9	6	0.93
	C 8.2§		5	0.89
	C 8.4		˙6	0.91

TABLE IV—*Concluded*

	Sample No.	Time	Volume	pH ($\pm$ 0.01)
		hrs.	*cc.*	
Experiment C 5.‖	C 5.2	Overnight	20	1.14
Dog Q	C 5.3	"	20	0.92
	C 5.4	4	8	1.02
	C 5.6	6	3	0.94

* These samples were obtained by adventitious retention of juice, following accidental inversion of the mucosa. Throughout this table, time represents the number of hours after ingestion.

† These samples were collected while the animal was kept on its right side, so that the juice might accumulate in the pouch.

‡ These samples were collected after the badly eroded area had been healed with simultaneous formation of sphincter action. At the time Sample C 2.15 was taken, the animal showed first signs of a gastrointestinal disturbance which lasted for several weeks (see Experiment B 17). Sample C 2.21 was taken after the dog had recovered.

§ Samples C 8.2 *et seq.* were collected about a month after the C 4 series had been discontinued and about 3 months after operation.

‖ These samples were collected before a perforation developed in the septum between stomach and pouch. During the repair operation the pouch was converted to the Heidenhain type.

these three values was so very striking, that, in spite of the paucity of data, an effort was immediately made to modify the pouch operation in a such way as to afford sphincter action directly, without the formation of profuse scar tissue. After several failures, the technique described above (see "Preparation of gastric pouch dogs") was finally perfected.

Before that end was attained, however, an attempt was made to collect such retained juice in another way. Dog G had not yet developed sufficient scar tissue around the mouth of the pouch to hold the fluid within it. Therefore, after removing the dressing, the animal was allowed to lie on its right side. When in this position, the opening of the pouch was at a level higher than the body of the pouch and some fluid was retained. In spite of great leakage resulting from the dog's movements, it was occasionally possible to remove some juice by aspiration with a fine rubber catheter and a syringe. At no time was the volume thus obtained very great and, obviously, most of the fluid secreted during the experiment was lost. Of the four satisfactory experiments conducted in this way (Experiment C2, preliminary), two gave pH

values in perfect agreement with those of Experiment C 1.   The
others were higher, though far nearer the minimum values than
even the average of all pH values obtained in Series I.   The
significance of these two irregularly high values will become clear
later from a study of the data in Table V.

   With the regular retention of juice following the prevention of
erosion in this dog (Dog G) and the consequent formation of scar
tissue, the ease with which such samples were obtained was greatly
increased.   The animal could be permitted its freedom in the cage
with the dressing on, until it was desired to remove the pouch con-
tents.   The same procedure was followed with Dogs O and Q,
in both of which sphincter action was obtained as already de-
scribed.   In the case of Dog Q, shortly after Sample C 5.6 was
obtained, the animal developed a perforation of the septum be-
tween the stomach and the pouch.   In repairing the opening, the
nerve was cut in this region thus making the pouch one of the
Heidenhain type.   Thereafter, no postprandial juice could be
obtained although the response to histamine was normal (see
Experiments B 27 through B 29).

   Results with these three dogs are listed in Experiments C 2,
C 4, C 5, and C 8.   Examination of the sample numbers in Table
IV will reveal that the list of consecutive samples is not complete.
The samples corresponding to the missing numbers were either
collected under special conditions for other purposes or else were
discarded because of the presence of blood or excessive mucin.
The pH values thus obtained show unmistakable clustering about
0.91.   A detailed analysis of the data reveals the following.   Of
the thirty-seven pH values listed in these four sets of experiments,
twenty-two (60 per cent) fall within the range of what we believe
may fairly be regarded as indicating the minimum pH established
in Series III; i.e., 0.91 ($\pm$ 0.02); four (10 per cent) are just beyond
this value.   The remaining eleven (30 per cent) vary between
0.96 and 1.55, with only two values being higher than 1.06.

   In view of the fact that about two-thirds of all these pH values
were at or near the minimum observed in the previous series, the
question arose as to the cause of the variation in the remaining
one-third.   Now, it was noticed that mucin clots[4] occurred to a

   [4] The term "mucin" refers specifically to the protein constituent of the
viscous "mucus" secretion.   The importance of this distinction in the
present work is developed below in the "Discussion."

much smaller extent in these samples than it did in those of Series I and II. On the other hand, in the experiments with the larger doses of histamine, it was observed that the first sample usually contained an appreciable amount of mucin while subsequent samples contained little or none. Although it was impossible to establish a quantitative correlation of mucin content with a lowered acidity, the frequency of concurrence of the two could not be ignored. It seemed almost certain that the extremely

TABLE V

Series IV. Discontinuous Collection Experiments. Pairs of Successive Postprandial Samples

	Sample No.	Time*	Volume	pH ($\pm$ 0.01)
		hrs.	*cc.*	
Experiments C 4 and	C 4.13	3	2.5	1.08
C 8. Dog O	C 4.14	4¼	5.3	0.91
.	C 4.19	3	12	0.97
	C 4.20	3	13	0.94
	C 4.21	4	8	0.95
	C 4.22	4	20	0.93
	C 8.1		3.5	0.98
	C 8.2		5.0	0.89
	C 8.3		2.0	1.27
	C 8.4		6.0	0.91

* The time value for the first sample of each pair is the number of hours after ingestion of food; that for the second sample is the time between the two collections.

low acidity of the first sample in each of the continuous collection experiments was due in part to an accumulation on the walls of the pouch of viscous mucus which could not be removed by simple drainage. This *accumulated* mucus must not be confused with that which may be secreted during the experiment. In order, therefore, to determine whether the high pH values of Series IV can be ascribed to admixture of such a retained substance, the experiments of Table V were performed.

It will be noticed from Table V that the samples were collected

in pairs. The technique was identical with that of the previous experiments except for one detail. Instead of taking one sample each day, two such were taken at intervals of several hours as indicated. The first sample obviously contained not only mucus secreted since the meal but a very large part of that secreted during the previous 18 to 24 hours. The amount of mucus present in the second sample was only that secreted since the collection of the first sample, a few hours previous. In each of the five experiments recorded in Table V the value for the second sample is invariably near the minimum pH; the first is always higher than it. Now, in collecting the samples of Experiments C 2, C 4, C 5, and C 8 reported in Table IV, such a preliminary "washing out"

TABLE VI

Distribution of pH Values in All Four Series of Experiments

Series No.	Table No.	Average maximum rate	Average of all pH values	Distribution of pH values (No. of samples and per cent of total within each range)			
				Total	0.91 (± 0.02)	0.94–1.08	Above 1.08
I	I	1.8	1.37	38 (100)*	0 (0)	13 (34)	25 (66)
II	II	2.5	1.02	23 (100)	1 (4)	17 (74)	5 (22)
III	III	5.2	0.97	57 (100)	37 (65)	11 (19)	9 (16)
IV	IV	Equivalent to Series I	0.96	37 (100)	22 (60)	13 (35)	2 (5)

* The per cent values are given in parentheses.

of the pouch was not practiced as a routine procedure, although it happened several times as a result of the accidental loss of a pouchful of juice due to the pressure of the secretion being great enough to force the sphincter. Therefore the lack of this "washing out" of the pouch in all probability accounts for the irregularly low acidity values recorded in Table IV.

Comparison of the Four Series of Experiments

Table VI presents a study of the distribution of the pH values in the four series of experiments just described. The third column contains the average, for the entire series, of the highest secretory rate tabulated for each experiment. As such, this figure is a crude measure of the relative rate of secretion under the conditions of each series. Obviously, no corresponding value is

possible for Series IV (*discontinuous collection experiments*) but independent observations have shown that the total volume of juice secreted with any one diet and dog is independent of the collection technique—as might be expected. In the fourth column are listed the averages of all the pH values within each series of experiments. Since pH is a logarithmic function of the acidity, one must be careful not to attach too much significance to these arithmetical means. Nevertheless, such calculations are of much value as a qualitative indication of group differences. Finally, in the fifth and subsequent columns of Table VI there is shown the distribution of the individual pH values within each series. The three divisions chosen are 0.91 ±0.02, 0.94 to 1.08 (selected only because 1.08 is the pH of 0.1 N HCl), and values greater than 1.08. Such an organization of data affords a ready survey of the relative tendency of each series toward attainment of the minimum pH value.

A comparison of Series I, II, and III indicates a progressive rise in average acidity corresponding to the rise in average maximum rate of secretion. Apparently, therefore, the series behavior is identical with the commonly observed parallelism of these two variables (in continuous collection experiments of this sort). Similarly, the distribution data show a regular shift in the pH interval containing the greatest proportion of pH values. In Series I, two-thirds of the values are less acid (higher) than 1.08, with none at the minimum. Whereas only one value is at the minimum pH in the next series, 74 per cent are now in the second division. With the further increase in average acidity in Series III, the bulk (65 per cent of the values fall within the minimum range. The distribution between the other two divisions is about equal. In the last series, the proportion of values at the minimum pH is almost the same as in Series III; only here, most of the remaining values (all but two) fall in the 0.94 to 1.08 division. In both Series III, a *continuous collection* series, and Series IV, a *discontinuous* series, the greatest proportion of the pH values falls within the first range, and no values smaller than this were observed. It is evident, therefore, that the acidities do not exhibit a distribution of the random sort. In other words, *there is a definite upper limit to the acidity attainable in these experiments.*

On the other hand, the secretory rates of the two postprandial

series, Series I and IV, representative of the two methods of collecting the juice, were about the same. Therefore, *the attainment of this upper limit is not caused by a high secretory rate*, but by some other factor. Replacing the older continuous collection method by the sphincter technique served to reduce the magnitude of this factor or of its influence. This other variable operating to reduce the acidity is apparently the flow of mucus secretion. In Series I a rubber catheter was held in the pouch, rubbing the mucosa and causing a slight but perceptible flow of mucous protective (?) fluid throughout the experiment; in the discontinuous or retained juice series, Series IV, this constant though mild irritant was absent. Consequently different amounts of this "contaminant" of the secretion of the parietal cells were present in the two cases resulting in different acidities.

In the experiments of Series I and II there can be no doubt of a definite parallelism of acidity with secretion rate. Traces of mucus which adhered to the pouch wall before the experiment was started had undoubtedly been washed out by the end of the second sample. But the correlation may persist for several hours thereafter, as in Fig. 2, Experiment B 5. Throughout these experiments, therefore, we believe there occurs a slight flow of mucus or other alkaline fluid under the stimulus of a fairly constant irritant, *i.e.*, the catheter. Consequently, with an increase in flow of acid secretion, the surface between catheter and mucosa is lubricated to some extent thus reducing the amount of irritation and therefore the absolute amount of alkaline secretion present. In Series III, the flow of parietal secretion is so great as to reduce the friction to a minimum. In this way, there arises the large number of pH values which lie within the minimum range for this series.

DISCUSSION

The data just presented, in our opinion, offer considerable support for the view that when the parietal cells elaborate hydrochloric acid they pour out this substance at a definite concentration, determined probably by the osmotic equilibria of the animal. In support of this view and in opposition to the hypothesis of Rosemann (1907, 1920), Foster and Lambert (1908, *a* and 1908, *b*), MacLean and Griffiths (1928), and others, we have adduced two argu-

ments, as follows: (1) By the analysis of the data presented in
Table VI, it has been shown that *there exists a definite maximum
acidity* of pouch juice attainable under these experimental con-
ditions.   (2) This maximum acidity can be attained without any
significant change in the rate of secretion, as shown by the *dis-
continuous collection technique* employed in Series IV.   On the
other hand, when the maximum acidity has once been attained in
a continuous collection experiment, as in Series III, this value may
persist in spite of a marked drop in the secretory rate.   Conse-
quently, *variations in rate of secretion are not of necessity accom-
panied by corresponding changes in acidity.*

It is highly unlikely that the constant maximum acidity ob-
served in this study represents an upper limit to the varying de-
grees of hydrolysis of neutral chlorides, as Rosemann postulated.
On the contrary, this constancy is, in our opinion, more directly
indicative of a parietal secretion of constant HCl content.   The
value of this normal maximum has been confirmed by additional
measurements made on eight other Pavlov pouch dogs prepared
more recently by both of us working independently.[5]

Further confirmation is afforded by the corresponding data for
humans and cats recorded in the literature.   In cats, the highest
value reported by Carlson, Orr, and Brinkman (1914) is 0.55 per
cent (pH 0.91, 0.151 N).   Gamble and McIver (1928) working
with this same species found that the total ionic content was
stationary at 0.165 N.   Observations on humans are similar.
Frouin (1899) quotes Seeman to the effect that the total acidity
is about 0.6 per cent (pH 0.88, 0.160 N).   Menten (1915) found pH
values as low as 0.92 (0.147 N) in Mr. V., a gastric fistula case,
while Kaznelson (1907) obtained similar values as acid as 0.14
N (0.51 per cent, pH 0.94).   Boldyreff (1915) cites pH values 0.92
to 0.94 (0.147 to 0.140 N).   Delhougne (1926) reported total
acidity values as high as 0.150 to 0.160 N.   Also, Rosemann (1920)
cites a number of investigators—including Sommerfeld (1905),
Bickel (1906), and Umber (1905)—as having found total acidity
values of 0.55 to 0.60 per cent (pH 0.91 to 0.87, 0.164 to 0.151 N).
The agreement of all these values with the corresponding values
for cats and with our maximum acidities for dogs is such as to

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[5] We are indebted to Mr. Alfred Gilman for securing numerous data from
some of these additional animals.

leave no doubt of the existence of some factor common to the intracellular secretory mechanisms of all three species; in all probability, this is the osmotic pressure of the tissue fluids,

That mucus is not the only agent effective in reducing the acidity, however, is quite certain. While in general, retained samples of the lower pH values show less mucin than do those of higher pH (or lower acidity), this is far from being universally true. Likewise, in samples collected by continuous drainage, the absence of a quantitative relation between mucin clot and acidity has also been noted. Such observations suggest that other agents effective in reducing acidity must be sought. Numerous investigators have already hinted at the presence of another fluid, the "Verdünnungssekretion." Whether the epithelial detritus and a lymph transudate play any important rôle in the process we cannot say at present. On the other hand, the secretion from the peptic cells does unquestionably play a part, though as yet we have no notion of its magnitude. It is not impossible that what different investigators have been calling the "Verdünnungssekretion" really consists simply of the products of both the mucus- and pepsin-forming cells.

The chemical process by which these non-parietal secretions effect a reduction of gastric acidity merits some consideration. Foster and Lambert (1908, b) have argued that, granting that mucus does occur in the gastric juice as collected, its addition to the HCl would affect the free acidity but not the total as determined by titration with NaOH and phenolphthalein. This is so, they maintain, because the sodium ion will replace the mucus group in the mucus-HCl compound as it does the hydrogen ion in HCl, since a strong base will always replace a weaker one. The fallacy of the argument arises from a confusion of the entire mucous secretion with one of its components, mucin. It has been shown by a number of investigators (Gamble and McIver, 1928; Grechen, 1909) that the product of the mucus cells is a highly viscous liquid containing most of the inorganic constituents characteristic of plasma as well as the protein, mucin. Consequently, it contains a fairly large proportion of alkali bicarbonates, the addition of which to the parietal HCl results in an absolute loss of acid in the form of CO_2. Thus, not only will there be a decrease in the free acidity but in the total as well when the mucus secretion mixes with

that of the parietal cells. Simultaneously, alkali chloride and an exceedingly small amount of protein-HCl compound will be formed. The latter may behave in the manner described by Foster and Lambert, but the variations in acidity which concern us here probably result from the reaction with bicarbonate.

This loss of acid as CO_2, however, accounts only in part for the reduction in acidity. The addition of mucous secretion, even were it free of bicarbonate, would reduce the acidity of the original HCl solution by a simple dilution process. Similarly, even if the other contaminants, like the pepsin secretion, do not contain appreciable amounts of bicarbonate, they must exert a very significant influence by this dilution effect alone. It may be argued that such a dilution process should have an equally marked effect on the chloride ion concentration of the gastric juice. The diluent, however, contains a very high concentration of neutral chlorides and the pH is near that of the neutral point. Consequently, apart from any true neutralization by bicarbonate, etc., the effect of dilution in reducing gastric acidity will be many times as great as its effect in diminishing the total chloride.

There remains for consideration the bearing of these observations on the opposing views of Rosemann; namely, that (1) not the HCl content but the total chlorine is the constant factor, and (2) the decrease in acidity during the latter part of the digestive period from its previously high value is contradictory to Pavlov's theory, because all of the mucus should have been washed out during the early part of the experiment. Inasmuch as no data for total chlorine are presented in this paper, a consideration of the first of these points is reserved for a later communication. Regarding the second contention, however, it is apparent that Rosemann entirely neglected the possibility of a continued secretion of mucus throughout the experimental period. When this factor is minimized, as in the histamine experiments with a high secretory rate, so that only the initial retained mucus plays a major rôle, the results are entirely as Rosemann demanded of Pavlov's theory; i.e., no drop in acidity occurs after the initial rise (see Fig. 3, Experiment B 27) until the fluid collected contains a relatively large amount of mucus and but a small amount of the parietal secretion.

SUMMARY

A method has been described whereby accessory stomach pouches in dogs can be provided with sphincters. With the aid of such sphincter action it was possible to collect gastric juice secreted in the absence of a solid collecting device—*the discontinuous collection technique* here used—thus minimizing the amount of mucus secretion arising from irritation of the mucosa by such a solid body. These dogs have also been used for *continuous collection experiments.*

The observations of Pavlov regarding the parallelism of acidity and secretory rate for postprandial gastric secretion in pouch dogs have been confirmed (Series I). Furthermore, it has been found that the same relation obtains when histamine is used as a secretory stimulant, provided the rate of secretion is not significantly greater than that obtained when food (Series II) furnished the stimulus. On the other hand, when a much higher rate was induced, by the use of a larger dose of histamine, this parallelism disappeared. Instead, the acidity rose to a maximum value at which it remained constant until the rate of flow of juice had again fallen to a low level (Series III). In most cases, this velocity value was so low as to correspond to a preponderance of mucus; *i.e.*, the secretion of acid had almost ceased.

Maintaining the general conditions of Series I, postprandial experiments were next carried out by the discontinuous collection method (Series IV). The average acidity obtained by this technique was very much greater than that obtained by the continuous collection method, and practically the same as in Series III. It was also shown that, if retained mucus be "washed" out of the pouch by discarding the first sample of postprandial juice, subsequent samples invariably possess acidities at or very near the maximum. A similar attainment of the maximum occurs in Series IV because of the initial absence of the object which causes this irritation, the catheter.

The value of this maximum acidity in pH units was 0.91 ±0.02. Data are presented showing this to be constant for five mongrel dogs of widely different physical characteristics, under a variety of conditions. Subsequently, these results were confirmed by the data from eight additional animals. Confirmation by data in the

literature for cats and human beings was also pointed out.[6] Such
agreement among the three species is regarded as supporting the
view that the unaltered secretion of the parietal cell is isotonic
with the blood, the osmotic pressure of which is about the same in
all three species, and that the true acidity of this secretion is
determined by the osmotic equilibria of the animal.

The deviations from this maximum are ascribed to contamina-
tion of the secretion of the parietal cell by that of the mucus-
forming and peptic cells. A lymph transudate and epithelial
detritus may perhaps play a minor part.

The authors advance the view that the chemical mechanism by
which the acidity of gastric pouch juice is reduced depends upon
two factors: (1) a simple dilution by fluids (mucus, peptic secre-
tion, etc. (?)) containing a large concentration of neutral chlorides,
as a result of which acidity is reduced much more than is the total
chloride content; (2) an actual neutralization by the bicarbonate
in at least one of the fluids, as a result of which there is a further
marked reduction in total acidity. Mucin probably plays only a
minor rôle in this second process.

These results, we believe, constitute ample confirmation of the
Heidenhain-Pavlov hypothesis regarding the constant acidity of
the secretion of the parietal cells. At the same time, by demon-
strating the possible independence of acidity and rate of secretion
they present a fundamental incompatibility with the opposing
theory of Rosemann and his supporters.

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[6] This minimum pH value, however, does not of necessity imply the com-
plete elimination of· mucus. Recently, one of us (F. H.) has repeated these
experiments using a more sensitive method for measuring acidity; i.e.,
a micro titration method with a carefully controlled end-point. In such
cases, what appears to be constancy by the less delicate pH method was
found to be a typical curve with a maximum acidity corresponding to the
maximum secretory rate. The curvature was so slight, however, as to
remain constant within the limits set in the present investigation. This
minute fluctuation is exactly what one would expect if the mucous secretion
were not quite entirely eliminated.

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THE DISTRIBUTION OF ELECTROLYTES BETWEEN SERUM AND THE IN VIVO DIALYSATE*

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Knowledge regarding the state of the electrolytes in the serum is necessary for a satisfactory understanding of the formation and maintenance of the various normal and pathologic fluids in the body, such as the lymph, cerebrospinal fluid, aqueous humor, synovial fluid, or edema and ascitic fluids. Various methods have been devised for the study of the chemical equilibrium between these various fluids and the blood stream and attempts have been made to determine the condition of the ions present.

Michaelis and Rona (18), and Rona and Takahashi (22) introduced the use of "compensation dialysis" for the study of the state of various crystalloids in the plasma. At equilibrium the latter found that the concentration of calcium in the serum was greater than that in the outside solution, and the difference was assumed to be due to the presence in the serum of calcium in a colloidal or non-diffusible form. This method has since been extended to a study of the other anions and cations in the serum. The technique has been improved to permit the control of the hydrogen ion concentration and carbon dioxide tension. Mestrezat and Ledebt (17), on the other hand, filled collodion sacs with saline solution and placed them in the abdominal cavity of rabbits. The sacs were removed several days later and the contents were analyzed. They considered that equilibrium had been attained, and that the sacs contained a true dialysate of the

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blood stream. However, this method provides small amounts of dialysate and does not permit the simultaneous study of the blood and dialysate. Numerous investigators have studied the composition of the ultrafiltrate of blood serum and Brull (3) has developed a method for obtaining an ultrafiltrate *in vivo*.

The majority of previous studies of the problem have failed to give satisfactory information regarding the state of the electrolytes in the blood stream. Frequently the experiments have been too few, or the analytical methods have not been sufficiently accurate, either to rule out the effect of technical error or to show the magnitude of the experimental variation present. Experiments with dialysis or ultrafiltration *in vitro* are open to question, since either there was no control of such factors as hydrogen ion concentration or carbon dioxide tension or the experimental conditions chosen may not have represented those physiologically present within the body. The *in vivo* experiments of Brull (3) and Mestrezat and Ledebt (17) furnish only small amounts of fluid for investigation.

We believe that our method of compensation dialysis (10, 11) *in vivo* is free from these various objections. It not only furnishes a method for studying the equilibrium between a physiologic saline solution and the arterial blood stream when the two are separated by a relatively semipermeable collodion membrane, but also provides a method for making this study under normal and strictly physiologic conditions. We have sought therefore to determine the electrolyte equilibrium so attained, and to use the composition of the dialysate as a basis for the study of the cerebrospinal fluid and of edema and ascitic fluids.

Methods

Experimental—The details of the experimental procedure will be reported. Dogs were used in all experiments, the majority of which were done under narcosis induced by sodium isoamylethyl barbiturate. This, however, was largely a matter of convenience, for identical results were obtained in control experiments, in which local anesthesia was employed. Heparin was used as an anticoagulant. This substance may affect the colloidal state of the plasma but control experiments showed that it was without effect on the concentration of the various electrolytes in the blood. At

the beginning of the experiment a dialyzing tube was connected to each femoral artery and vein. The jackets of the tubes were then filled with modified Ringer's solution of such composition that the initial concentration of each, with respect to the blood serum, was hypertonic in one tube and hypotonic in the other. The dialyzing fluids were analyzed at intervals during the experiment and the progress to equilibrium was followed. Dialysis was usually continued for an additional hour thereafter to insure the completeness of the final equilibrium; the entire experiment lasted for 4 to 7 hours. At the end of that time, samples of blood and of the two dialysates were taken for analysis. Throughout the experiment, respiration and secretion of urine were apparently unaffected. Adsorption by the dialyzing membrane, with resultant disturbance in the final equilibrium, must be guarded against in experiments *in vitro* but in the experiment *in vivo* there is a continuous circulation through the dialyzing sacs. Furthermore, the volume of the dialysates was small in comparison to the total blood volume of the animal, and the normal regulatory mechanisms of the organism were available to compensate for the effects of any passage of salts or fluid in or out of the dialyzing sacs. The dialyzing apparatus formed a closed system, so that such various factors as temperature, carbon dioxide tension, and hydrogen ion concentration were automatically controlled. Because of this control of various possible sources of error, we believe that this method of *in vivo* dialysis gives a more accurate indication of the true equilibrium than the various methods of *in vitro* dialysis previously proposed.

Analytic—Samples of blood were taken under oil directly from the arterial cannula. Sufficient heparin was used to prevent coagulation in the dialyzing tubes, but not enough completely to inhibit coagulation of blood, so that when the tubes were centrifuged immediately, the corpuscles were thrown down before coagulation of the plasma occurred. The samples were then put on ice until coagulation was complete, when they were recentrifuged and the serum was removed. In this way redistribution of the different ions between the serum and corpuscles if it occurred at all was kept at a minimum. In the last five experiments, samples of blood were taken over mercury and the serum was separated without exposure either to air or oil. The hydrogen ion

concentration and carbon dioxide content of such serum was the same as that of specimens taken in the ordinary manner. Samples of the dialysates were collected over mercury without exposure to air. They were completely protein-free, according to the usual tests.

In the first ten experiments, the concentration of chlorides was determined by the method of Van Slyke (25); in the last five, that of Wilson and Ball (30) was used. We did not find any difference between the two methods when applied to dog serum and there is no difference between the two sets of experiments. Similar observations have been reported by Eisenman (7). Phosphates were determined by the method of Fiske and Subbarow (8).

Carbon dioxide was determined by the manometric method of Van Slyke (26). Sodium was determined by the gravimetric method of Kramer and Tisdall (15). Jena glass crucibles with fused-in filtering disks were used in filtering the precipitate of pyroantimonate. The use of these crucibles, and of a fixed and rigid technique in washing the precipitate, permitted the determination of sodium with an average difference between duplicates of 4.5 mg. (1.8 per cent). Control experiments on ashed specimens of serum gave the same results as with the method of direct precipitation. Calcium was determined by the Clark-Collip (4) modification of the Kramer-Tisdall (15) procedure, with 2 cc. samples. Magnesium was determined by precipitation as the ammonium magnesium phosphate, and the determination of the phosphate in the precipitate made by the Fiske-Subbarow (8) method. The direct precipitation of potassium as the cobalti-nitrite gives high values in the serum of dogs. In the first ten experiments potassium in the serum was determined in the trichloroacetic acid filtrate according to Kerr's (14) modification of the Kramer-Tisdall (15) procedure. Later, the serum was ashed directly by repeated evaporation to dryness with nitric and hydrochloric acids, and the potassium was precipitated in the ashed solution. Similar results were obtained by the two methods. Because of the excess of potassium in the corpuscles, examination with a hand spectroscope was used to rule out all traces of hemolysis in the samples of serum. Wherever possible, all analyses were made in duplicate. The analytic methods were rigidly controlled by the analysis of known solutions similar in composition to the serum.

TABLE I

Composition of Blood Serum and the in Vivo Dialysate

Na, K, Ca, Mg, chloride, and P values are measured in mg. per 100 cc.

Experiment No.	Serum water (gm. per 100 cc.)	Serum protein (per cent)	Na Serum	Na Dialysate A	Na Dialysate B	K Serum	K Dialysate A	K Dialysate B	Ca Serum	Ca Dialysate A	Ca Dialysate B	Mg Serum	Mg Dialysate A	Mg Dialysate B	Cl (NaCl) Serum	Cl (NaCl) Dialysate A	Cl (NaCl) Dialysate B	CO₂ Serum	CO₂ Dialysate A	CO₂ Dialysate B	PO₄ (P) Serum	PO₄ (P) Dialysate A	PO₄ (P) Dialysate B
1	93.84*	6.45	369	333	334	17.6	15.1	15.4	11.2	6.8	6.7	2.7	1.5	2.0	651	656	661				5.8	3.0	3.9
2	93.76*	6.55	356	336	343	16.2	10.3	11.0	10.3	6.7	6.3	2.4	1.9	2.0	669	727	728				5.1	5.0	3.8
3	93.10*	7.37	362	362	338	17.7	14.8	14.7	10.3	6.8	5.5	1.2	0.6	0.7	696	746					2.9	2.2	6.6
4	93.68*	6.65	310	309	312	20.0	14.8	14.5	9.4	6.8	5.7				655	710	705				7.2	6.1	4.1
5	94.02*	6.23	358	352	339	15.6		11.2	12.4		6.7				670		711				5.0		
6	92.95*	7.55	376	352	346		17.9	17.5		6.8	7.3		1.3		701	750	757						
7	93.61†	6.70	363	348	347	17.3	11.5	12.9	10.8	6.4	5.8	1.9	1.5	1.1	727	766	768	45.6	48.1	48.3	5.6	5.2	6.0
8	93.72†	6.60	350	343	342	21.8	15.4	15.4	10.8	5.4	5.8	2.4	1.4	1.3	714	770	770	32.6	37.1	39.2	3.0	3.0	3.4
9	93.44*	6.95	345	331	336	17.3	12.2	12.4	11.6	6.2	5.5	2.6	2.4	1.7	640	719	713	48.2	51.7		6.3	5.6	6.1
10	93.81*	6.48	359	332	341	16.2	16.0	14.8	10.4	7.0	5.8	2.5	1.0	2.5	671	720	719	48.6	53.0	53.0	4.3	4.3	4.4
11	93.97†	6.36	340	347	345		16.5	16.1	9.3	6.7	6.5	1.2	1.0	1.1	621	690	692	54.9	60.5	60.8	4.2	4.9	5.1
12	94.73†	5.34	341	348	319	16.4	15.4	15.6	8.8	5.7	5.7	1.6	1.0	1.2	605	673	675	51.9	54.2	57.8	3.6	3.9	4.1
13	92.90†	6.01	338	326		17.8	15.7	20.1	9.6	6.3		2.2	1.6		635	670	675	47.8	54.9		4.1	4.2	
14	93.50†	6.54	325	312	318	23.6	23.2		9.1	6.0	5.5	3.3	1.6	1.7	644	700	696	48.3		50.6	7.4	4.5	4.0
15	94.07†	5.60	340	321	325	15.1	13.1	13.5	11.7	7.8	7.9	2.8	1.8	1.9	600	655	659		50.9	53.7	6.1	6.1	6.2
Average	93.67	6.50	348	333	334	17.9	15.1	14.7	10.4	6.5	6.2	2.2	1.5	1.6	660	710	709	47.24	51.3	51.9	5.0	4.5	4.8

* Calculated from the formula of Van Slyke, Wu, and McLean (28).
† Determined directly.

Results

Fifteen experiments were made in all, the results of which are shown in Table I.   The composition of the serum in these animals corresponded closely to the normal values previously reported both for the serum of normal men and of dogs.   In the majority of the experiments equilibrium was obtained in both dialysates and although the composition of the two solutions was very diverse at the beginning of the experiment at its termination the composition of the two dialysates agreed within the limits of error of the analytic methods used.   Particularly good agreement˙ was obtained in the case of both the sodium and the chlorides.

The concentration of the different cations was uniformly greater in the serum than in the dialysate.   In only two of fifteen experiments was the sodium content of the dialysate equal to that of the serum and the average value was 96 per cent of that in the serum. The potassium content of the dialysate varied from 65 to 99 per cent of that in the serum with an average value of 80 per cent or 15 mg. for each 100 cc.   Since the experiments of Rona and Takahashi (22) it has generally been accepted that part of the calcium in the serum is combined with protein or exists in some other non-diffusible form.   This view is confirmed by the present experiments, for the average calcium content of the dialysate was 61.5 per cent of that in the serum, a value for the diffusible calcium in the serum of approximately the same magnitude as those generally reported in experiments in which this value has been determined by dialysis *in vitro*.   The dialysate also contained less magnesium than the serum.

To this extent these results confirm previously expressed views that part of the different inorganic bases in the serum is held in a non-diffusible form, probably in consequence of their combination with protein or adsorption on it.

The chlorides and carbon dioxide in the dialysate were uniformly higher than in the case of the serum.   In only one experiment did the chloride content of the two solutions approach equality and the average amount in the dialysate was 108 per cent of that in the serum.   The average carbon dioxide content of the dialysate was 110 per cent of that of the serum.

The distribution of the inorganic phosphates did not correspond to that of the chloride and carbon dioxide.   The dialysate con-

tained on an average 94 per cent of the amount of inorganic phosphates present in the serum. Apparently a small amount of the phosphates in the serum is held in colloidal or non-diffusible form, a view that has previously been expressed by others as a result of studies on renal secretion and the glomerular filtrate.

Comment on Experimental Data

A general consideration of present day views concerning the physicochemical characteristics of protein solutions need not be given here. There is no general agreement among the various and opposing view-points presented. The significance of the so called membrane equilibrium is generally recognized, although the explanation of the observed facts may vary widely. The dialysates studied in the present experiments were obtained by means of a semipermeable membrane; consequently it has seemed advisable to present the results primarily from the standpoint of the Donnan (6) theorem. Various workers have studied the state of the electrolytes in the blood serum and the equilibrium between the serum and the various body fluids. The extensive literature on this subject is given by Brull (3) and need not be repeated here.

A study of the equilibrium between the serum and the dialysate is best made on the basis of the data given in Tables II and III. In Table II, the various constituents are expressed in milliequivalents for each kilo of water present in the serum or dialysate. In the first ten cases the amount of water present in the serum was calculated from the formula of Van Slyke, Wu, and McLean (28), which is: $W = 99.0 - 0.8\ P$. W is the water content of the serum and P the percentage of protein. We have verified this formula in a large number of cases and can confirm its accuracy. The water content of the serum was determined directly in the last five cases. The concentrations of the various constituents of the dialysate were calculated on the assumption that the water present was uniformly 99.0 gm. for each 100 cc. of dialysate. The equivalent bicarbonate was calculated on the assumption that one-twenty-first of the carbon dioxide content of the serum represented free carbonic acid. This method of calculation does not take the variation in the hydrogen ion concentration into account but the latter was relatively constant, and this omission does not introduce significant error into this calculation. The

TABLE II

Electrolyte Equilibrium between Serum and the in Vivo Dialysate

Values are measured in milli-equivalents per kilo of water.

Experi-ment No.	Serum water (gm. per 100 cc.)	Serum pro-tein (gm. per 100 gm. H₂O)	Na Serum	Na Dial.	K Serum	K Dial.	Ca Serum	Ca Dial.	Mg Serum	Mg Dial.	Cl Serum	Cl Dial.	Bicarb. Serum	Bicarb. Dial.	P Serum	P Dial.	Total cations Serum	Total cations Dial.	Total anions Serum	Total anions Dial.	Cation excess Serum	Cation excess Dial.	Base proteinate by difference	Base proteinate per 1 per cent protein (m.-eq.)
1	93.84	6.87	170.8	146.5	4.81	3.94	6.0	3.4			118.6	113.7			3.6	2.0	183.3	155.1						
2	93.76	6.98	165.2	149.3	4.42	2.76	5.5	3.3	2.4	1.4	122.0	125.6			3.2	2.9	177.5	156.7						
3	93.10	7.92	169.1	148.5	4.88	3.81	5.5	3.1	2.1	1.6	127.9	128.8			1.8	1.7	181.6	157.0						
4	93.68	7.10	144.4	136.6	5.47	3.79	5.1	3.1	1.1	1.0	119.7	122.2			4.5	3.7	156.1	144.1						
5	94.02	6.62	165.6	149.0	4.25	2.89	6.6	3.4			121.8	122.7			3.1	2.4	178.2	156.6						
6	92.95	8.13	175.6	153.3	4.57			3.5			128.9	130.2					187.8	162.6						
7	93.64	7.15	169.1	152.5	4.73	3.15	5.8	3.1	1.6	1.0	132.8	132.4	21.0	20.8	3.5	3.2	181.2	159.8	157.3	156.4	23.9	3.4	20.5	2.83
8	93.72	7.04	162.6	150.3	5.96	3.98	5.8	2.8	2.1	1.1	130.2	132.9	15.0	16.5	1.9	1.1	176.5	158.2	147.1	151.3	29.4	6.9	22.5	3.20
9	93.44	7.44	160.4	146.5	4.96	3.18	6.3	2.9	2.3	1.1	117.1	123.6	22.6	22.4	4.0	3.4	174.0	153.9	143.3	149.2	30.7	4.7	26.0	3.49
10	93.81	6.91	166.9	147.5	4.23	3.99	6.1	3.2	2.2	2.0	122.0	124.2	22.2	22.9	2.7	2.5	179.6	156.7	147.1	149.7	32.5	7.0	25.5	3.69
11	93.97	6.77	156.9	151.9	4.21	4.01	4.9	3.3	1.1	0.8	112.8	119.3	24.0	26.1	2.6	2.9	167.8	160.2	139.4	148.4	28.6	11.8	16.8	2.48
12	94.73	5.64	156.5	151.5	4.42	4.01	4.6	2.9	1.4	0.9	109.2	116.3	23.4	24.2	2.2	2.3	166.9	159.4	134.8	143.0	32.0	16.4	15.7	2.78
13	92.90	6.47	157.8	143.5	4.88	4.06	5.0	3.6	2.0	1.3	116.8	116.1	22.0	23.7	2.5	2.4	169.7	152.0	141.4	142.3	28.3	9.7	18.6	2.80
14	93.50	7.00	150.8	138.4	4.25	3.58	4.8	2.9	2.9	1.4	117.6	120.5	21.5	21.9	4.6	2.5	164.5	148.1	143.8	144.9	20.7	3.2	17.5	2.50
15	94.07	5.95	157.0	142.0	4.09	3.41	6.2	3.9	2.5	1.6	108.9	113.5	22.0	22.0	3.8	3.6	169.8	150.9	134.7	139.2	35.1	11.7	23.4	3.93
Aver-age...	93.67	6.93	161.9	147.0	4.90	3.82	5.56	3.22	1.9	1.3	120.4	122.8	21.46	22.20	3.1	2.6	174.3	155.4	145.0	147.7	29.3	7.7	21.6	3.12

inorganic phosphate concentration was converted into its equivalent base-binding power at pH 7.4 (1.8 × the millimolal concentration of HPO_4 (4)).

The Base-Binding Power of the Serum Proteins—Various determinations have been made of the amounts of base bound by the serum proteins, and formulas for the calculation of this value

TABLE III

Ratio between Electrolyte Concentrations in Serum and Dialysate

Experiment No.	$\dfrac{\text{Na in dialysate}}{\text{Na in serum}}$	$\dfrac{\text{K in dialysate}}{\text{K in serum}}$	$\dfrac{\text{Ca in dialysate}}{\text{Ca in serum}}$	$\dfrac{\text{Mg in dialysate}}{\text{Mg in serum}}$	$\dfrac{\text{Cations in dialysate}}{\text{Cations in serum}}$	$\dfrac{\text{Chloride in serum}}{\text{Chloride in dialysate}}$	$\dfrac{\text{Bicarbonate in serum}}{\text{Bicarbonate in dialysate}}$	$\dfrac{\text{P in serum}}{\text{P in dialysate}}$	$\dfrac{\text{Anions in serum}}{\text{Anions in dialysate}}$	Theoretic Donnan ratio
1	0.858	0.820	0.568		0.846	1.042		1.80		
2	0.904	0.625	0.591	0.600	0.883	0.972		1.10		
3	0.878	0.782	0.599	0.750	0.865	0.993		1.06		
4	0.946	0.693	0.669	0.500	0.924	0.980		1.21		
5	0.900	0.680	0.508		0.878	0.993		1.29		
6	0.873				0.866	0.990				
7	0.902	0.668	0.526	0.600	0.882	1.003	1.010	1.07	1.005	0.941
8	0.924	0.668	0.483	0.531	0.896	0.981	0.909	1.00	0.972	0.932
9	0.913	0.642	0.468	0.555	0.885	0.948	0.992	1.16	0.960	0.920
10	0.884	0.902	0.524	0.908	0.873	0.984	0.970	1.06	0.986	0.923
11	0.968		0.667	0.808	0.955	0.946	0.920	0.90	0.940	0.945
12	0.968	0.908	0.613	0.697	0.955	0.940	0.967	0.95	0.943	0.947
13	0.910	0.832	0.630	0.667	0.896	1.005	0.928	1.05	0.994	0.940
14	0.918	0.870	0.593	0.472	0.900	0.976	0.982	1.86	0.993	0.945
15	0.905	0.884	0.633	0.617	0.889	0.960	1.000	1.06	0.968	0.923
Average..	0.908	0.780	0.580	0.635	0.892	0.981	0.966	1.17	0.982	0.933

have been given by Van Slyke, Wu, and McLean (28), Hastings, Salvesen, Sendroy, and Van Slyke (12), Darrow and Hartmann (5), and Van Slyke, Hastings, Hiller, and Sendroy (27). These last obtained values considerably higher than those previously reported and pointed out the many technical difficulties in the direct determination of this value. The data in Table II indicate that the total base bound by protein may be considerably higher (25 per cent) than that calculated from the formula of Van Slyke,

Hastings, Hiller, and Sendroy (27). In these experiments the total cations and anions present in the solution have been determined by the summation of the analytic values for the individual constituents. This method admittedly may result in a summation of analytic errors, but in the case of the cations we have found, as has Whelan (29), that the sum of the cations so determined agrees accurately with the value for the total base directly determined by the method of Stadie and Ross (24).

The excess of bases over the determined acids in the protein-free dialysate varies from 3.3 to 16.3 milli-equivalents, with an average value of 7.6. Analysis indicates that sulfates and lactates will account for practically all of the undetermined acids in the dialysate. An excess of bases of more than 10 milli-equivalents was found in but three experiments, but in these the lactic acid content of both the serum and dialysate was increased. In the serum, on the other hand, the excess of determined base over acid varies from 20.7 to 35.1 milli-equivalents, with an average of 29.3. The undetermined diffusible anions may be assumed to have the same concentration in the serum as in the dialysate. This assumption may be accepted for the time being, for Perlzweig and Delruc (20) found values of corresponding magnitude on the direct titration of the organic acids in the serum, and other experiments of ours have indicated that the lactic acid content of the serum and dialysate is equal. If this value is added to the sum of the determined anions in the serum, then the extra cations present in the latter may be considered to represent the base bound by the serum proteins.

This indirect method of calculation in the present series of experiments indicates that the total base bound by protein varies from 15.8 to 26.0 milli-equivalents, with an average of 21.7. The average concentration of protein for each 100 gm. of water was 6.93 per cent. 1 per cent of protein in the serum, therefore, binds 3.13 milli-equivalents of base, a value considerably above those previously reported.

The Donnan Equilibrium— Several investigators, the most recent of whom were Hastings, Salvesen, Sendroy, and Van Slyke (12), have studied the distribution of the different inorganic ions between blood serum and ascitic fluid and between blood serum and salt solution, when dialysed *in vitro*, and have emphasized the

significance of the ratios between the molal concentrations of the different ions in the two solutions. The ratios obtained in this series of experiments are given in Table III. There were individual variations in the different experiments of the previous investigators, but the distribution ratios, which they found, fall within the limits of variation of the experiments in Table III. We have likewise found that when dialysis is carried out *in vitro*, with careful control of the hydrogen ion concentration and the carbon dioxide tension in particular, the total electrolyte distribution apparently is the same as that obtained by dialysis *in vivo*.

A study of the distribution ratios of the different ions between serum and a saline solution such as is obtained by dialysis, *in vivo* (Table III) shows characteristic differences in the behavior of the different ions. The distribution ratios for the chloride and bicarbonate ions, and for the total anions, are all of approximately the same magnitude. The slight differences between the ratios of chloride and bicarbonate have been attributed by others to factors inherent in the solutions, such as differences in the activity coefficients of these two ions, but the difference between these two ratios is not sufficiently great to be significant from a statistical point of view. In the case of the chlorides these ratios vary from 0.940 in Experiment 12 to 1.042 in Experiment 1, with a mean value of 0.9809 ± 0.0044, while if the total determined inorganic anions are considered, the ratios vary from 0.940 in Experiment 11 to 1.005 in Experiment 7, with a mean value of 0.9785 ± 0.0070. These values for the mean distribution ratio of chlorides or of total anions differ slightly from the value of 1.000 which would be required if there were a simple equilibrium between the electrolytes in the serum and in the dialysate. The differences in the mean ratios from unity, however, are greater than 3 times the probable error of these means and therefore are significant from the statistical point of view. The mean distribution ratios of the different cations, on the other hand, vary much more widely, the value ranging from 0.908 for sodium to 0.760 for calcium.

In accord with previous practice (12, 28) the theoretic Donnan ratios in the last column of Table III have been calculated on the assumption that the base proteinate is completely ionized. When the values for the base bound by protein are used, the values for the distribution ratio range from 0.920 to 0.947, with a mean value

of 0.933. The discrepancy between the theoretic ratio and the observed ratio in the case of either of the different anions or cations is too great to be explained as due to experimental error and indicates the difficulty in the application of the Donnan (6) theorem if it is assumed that the base proteinate is completely ionized.

It is generally recognized that part of the calcium in the serum is bound to protein in a non-diffusible combination. We have indicated that the other bases may be present in similar combination. Comparison of the experimental with the theoretic ratios suggest that if the Gibbs-Donnan law is to be applied to this system it must be assumed that only a part of the total base bound by protein is ionized and effective in the establishment of the membrane equilibrium.

The distribution ratios of the various anions are less variable than those of the various cations. These experimentally determined ratios may be used as an index to the effect produced by those factors responsible for the Donnan equilibrium. Granting this last assumption, a more detailed picture of the state of the bases in the serum can be obtained by substituting the experimental data in the appropriate equations derived for the Donnan (6) ratios.

$$r_{ef} = \frac{Cl_s^-}{Cl_d^-} = \frac{HCO_{3s}^-}{HCO_{3d}^-} = \frac{A_s^-}{A_d^-} = \frac{Na_d^+}{Na_s^+} = \frac{K_d^+}{K_s^+} = \frac{\sqrt{Ca_d^{++}}}{\sqrt{Ca_s^{++}}} = \frac{\sqrt{Mg_d^{++}}}{\sqrt{Mg_s^{++}}} = \frac{B_d^+}{B_s^+} \quad (1)$$

These equations, as pointed out by Donnan, are valid in the strict sense only when referred to the respective activities of the different ions. Without knowledge of these activities, the equations still hold as a first approximation when expressed in terms of the concentration of the different ions provided it is assumed that the diffusible salts in both serum and dialysate are either completely ionized or that their degree of ionization is the same.

The ionized sodium in the serum and the degree of ionization of the sodium proteinate present, then, may be obtained from the data in Table II, by using the following considerations.

Na_s = total sodium present in the serum as experimentally determined.
Na_{prs} = non-ionized and non-diffusible sodium proteinate in the serum.
Na_{prs}^+ = sodium ions in the serum paired with non-diffusible protein ions.
Na_{ds}^+ = sodium ions in the serum paired with diffusible anions.

Na_s^+ = total sodium ions in the serum.

Na_d^+ = total sodium ions in the dialysate assumed to be equal to the total sodium present and all paired with diffusible anions.

A_s^- = total diffusible monovalent anions in the serum.

A_d^- = total diffusible monovalent anions in the dialysate all paired with cations.

r_{sf} = the Donnan ratio.

Only the ionized constituents of the serum and dialysate enter into the Donnan equilibrium. Therefore:

$$r_{sf} = \frac{A_s^-}{A_d^-} = \frac{Na_d^+}{Na_s^+} = \frac{Na_d^+}{Na_{prs}^+ + Na_{as}^+} \tag{2}$$

$$Na_s^+ = Na_{prs}^+ + Na_{as}^+ = \frac{Na_d^+}{r_{sf}} \tag{3}$$

$$A_s^- = r_{sf} A_d^- \tag{4}$$

and by substitution of the sodium ions paired with diffusible monovalent (4) anions:

$$Na_{as}^+ = r_{sf} Na_d^+ \tag{5}$$

$$Na_{prs}^+ = Na_s^+ - Na_{as}^+ \tag{6}$$

The blood serum, however, contained some sodium bound to protein in a non-diffusible and possibly non-ionized state. Therefore

$$Na_{prs} = Na_s - Na_s^+ \tag{7}$$

and the degree of ionization of the sodium proteinate can be calculated from

$$\text{Per cent ionized} = \frac{Na_{prs}^+}{Na_{prs} + Na_{prs}^+} \times 100 \tag{8}$$

This same calculation may be applied to the other bases as readily as to sodium, although in the case of calcium and magnesium the square root of the molal concentrations must be used. The calcium and magnesium, however, constitute such a small proportion of the total bases that in this calculation the total base can be treated as though it consisted wholly of monovalent cations. The results of the above method of calculation, when applied to

the average values obtained in the present study, are shown in Table IV. The mean value for the distribution of chlorides between the serum and the *in vivo* dialysate was used as the most accurate index to the unequal distribution of ions produced by the presence of a Donnan type of equilibrium. The mean value of

TABLE IV

Calculated Distribution of Base in Serum

Donnan ratio $= r_{sf} = 0.9809$	Na	K	Ca	Mg	Total base
1. Total concentration in dialysate, m.-eq. (d)	147.0	3.82	3.22	1.3	155.4
2. Total concentration in serum, m.-eq. (s)	161.9	4.90	5.56	1.9	174.3
3. Concentration of ionized base in serum, m.-eq. $\left(\dfrac{d}{r_{sf}}\right)$	149.9	3.90	3.35	1.35	158.4
4. Concentration in serum as diffusible salts, m.-eq. $(r_{sf} \cdot d)$..............	144.2	3.75	3.10	1.25	152.4
5. Concentration in serum as ionized base proteinate, m.-eq. $((3) - (4))$.	5.7	0.15	0.25	0.10	6.0
6. Excess of base in serum over calculated concentration of ionized base, m.-eq. $((2) - (3))$........	12.0	1.00	2.21	0.55	15.9
7. Total base proteinate, m.-eq. $((5) + (6))$.............................	17.7	1.15	2.46	0.65	21.9
8. Degree of ionization of base proteinate, per cent $((5) \div (7))$.........	32	12	10	15	27
9. Base for each gm. protein, m.-eq. $((7) \div \text{protein})$.................	2.55	0.166	0.355	0.10	3.16
10. Total base proteinate, per cent of total base $((7) \div (2))$	10.9	23.5	44.2	34.2	12.6
11. Ionized base proteinate, per cent of total base $((5) \div (2))$............	3.54	3.06	4.5	5.2	3.4
12. Non-ionized base proteinate, per cent of total base $((6) \div (2))$....	7.4	20.4	39.7	29.0	9.1

0.9809 for the distribution of the chlorides, therefore, was taken as the standard r_{sf} used in determining the various combinations of the different bases shown in Table IV.

Table IV is of interest, for although it indicates that 17.7 milli-equivalents of sodium, 1.15 of potassium, 2.46 of calcium, 0.65 of magnesium, or a total of 21.9 milli-equivalents of base are com-

bined with protein, it also shows that approximately 6.0 milli-equivalents or only 27 per cent of this total may be considered as ionized base proteinate available for the establishment of a Donnan equilibrium. The ionized base proteinate varies from 32 per cent in the case of sodium to 10 per cent in the case of calcium of the total amount of each base that may be assumed to be in combination with protein.

There has been much discussion of the kind of combination between the different bases and the serum proteins. The evidence only shows that an appreciable amount of each of the different bases is held in the serum in a non-ionized, non-diffusible state and it may be that they are held in colloidal combination by other substances than protein. In the absence of direct evidence to the contrary, however, it is most likely that the proteins are the responsible materials. We wish to use the term "bound by protein" in the general sense without attempting to assign any value to the relative importance of various binding forces. This term has been used in the same way by Greenberg and Gunther (9), who pointed out that although at constant hydrogen ion concentration and protein content, the distribution of protein-bound and ionic calcium, with increasing calcium concentration, conformed to the Langmuir adsorption theorem, yet, that under the conditions of the experiments the Langmuir equation followed from the law of mass action.

Table IV indicates that for 1 per cent of protein in the blood serum 2.55 milli-equivalents of sodium, 0.17 milli-equivalents of potassium, 0.35 milli-equivalents of calcium, and 0.10 milli-equivalents of magnesium are in a combined form, giving a total of 3.16 milli-equivalents for the total base bound by protein. This represents the average value for the whole series of experiments. The results of individual experiments vary from 2.48 to 3.93 milli-equivalents for each per cent of protein.

We realize that these values for the base bound by protein do not permit a correction for shifts in the albumin-globulin ratio of the serum and may only be applicable to normal serum at normal hydrogen ion concentration. They are free, however, from some of the most serious objections to the previous methods of determining the base-binding power of the serum proteins; that is, the difficulty of dialyzing serum until completely base-free without

change in the proteins. They do not permit correction for the effect of changes in the hydrogen ion concentration or of the concentration of the different salts. Fortunately these values are all physiologic constants, which are so well maintained, at least in normal individuals, that the base-binding power of the serum proteins is likewise relatively constant.

The binding of approximately 10 per cent of the total sodium, 23 per cent of the potassium, 43 per cent of the calcium, and 32 per cent of the magnesium is of interest because of the suggestion of a Hofmeister series in this arrangement of the different cations. The results can equally well be explained on the basis of specific differences in the chemical affinity between the different cations and the various serum proteins or other substances. Certainly the behavior of the different cations is specific and characteristic in each case.

Free Dispersion Medium in Serum—In the consideration of the physicochemical properties of protein solutions such as blood serum an alternate method of treatment may be found in the discussion of those authors who have considered the solution as being composed of colloidal protein micelles suspended in the intermicellary fluid or free dispersion medium. The two are in equilibrium, yet the solution is to be considered essentially as a diphasic system. Augsberger (1) has summarized these views and has insisted that the composition of the dialysate or ultrafiltrate is a guide to the composition of the free dispersion medium or "lösende Raum" in the serum as contrasted with the colloidal micelle which occupies the "nichtlösende Raum." Various forces affecting the protein micelle such as positive or negative adsorption, electrostatic attraction or repulsion, the holding of water of hydration, and so forth, will influence the equilibrium between the micelle and the intermicellary fluid. Augsberger points out that according to this view the Donnan effect is a special case to be included in the more general relationships. The mathematical considerations involved in the calculation of the separate composition, and of the relationship between the two phases present in the serum are given in detail by him. These calculations are essentially the same as those previously used in calculating the values given in Table IV. In general it is assumed that the chloride or bicarbonate anions are present in the intermicellary fluid or the free dispersion medium

in the same concentration as in the dialysate. The distribution ratio of either of these anions then becomes an index to the proportion of water in the serum which is free and available as a solvent for the various electrolytes. The figures for the cations present in the serum as diffusible salts and for the bases combined with protein (Table IV) are unchanged, but are to be considered as representing the total amounts present in each phase rather than as concentrations in the solution as a whole.

This concept is acceptible, for various investigators have extended the Donnan theory of equilibrium to colloidal solutions in general, in the absence of a semipermeable membrane, by postulating that there is the same spacial arrangement of ionic constituents in the vicinity of a colloidal micelle as is obtained with a membrane interposed. Oakley (19) wrote

"The important condition for the establishment of a Donnan equilibrium is that one kind of an ion shall be prevented from diffusing where all the others can so diffuse. This condition is fulfilled in the case of surface ionization. Here the diffusion of one kind of an ion is prevented, not by a membrane, but by its forming a structural part of a large molecular aggregate. The idea of a membrane, is therefore to be replaced by the idea of a certain boundary near the surface of the particle beyond which the anions or cations, which are 'partners' to the colloidal cations or anions as the case may be, are unable to diffuse because of electrostatic attraction.

It is not necessary to regard this boundary as being the position of a well defined shell of ions, it is more natural to regard it as a 'diffuse' boundary in which the ions were widely distributed about a mean position of equilibrium. The distance of this boundary from the surface must be regarded as being the average position at which the electrostatic attraction of the ions by the layer of non-diffusible ions just counterbalances the resultant osmotic pressure which tends to make the ions concentrated near the surface diffuse evenly throughout the solution."

In blood serum the protein occupies only a relatively small proportion of the total volume of the solution, so that the greater portion of the solution is outside the mean position of this zone of electrostatic attraction of the protein micelles. As long as ultrafiltration is not carried so far as to cause any change in the protein micelles retained in the residue, the ultrafiltrate should consist of the free dispersion medium in the serum and so should be of constant composition. This fact has been verified experimentally by Augsberger (2) and Hirth and Tschimber (13), as well as by us.

From the practical standpoint it is convenient to consider blood serum as a diphasic system in which the dialysate may be considered as the equivalent of the intermicellary fluid or free dispersion medium present in the serum. With a knowledge of the composition of the serum and that of the dialysate, this assumption then permits the calculation of the relative amounts of the different constituents of the serum associated with the protein micelle and dissolved in the intermicellary fluid. This calculation is descriptive and of value in characterizing the solutions concerned. It does not give definite information regarding the forces such as chemical valences, electrostatic attraction, adsorption, and so forth, determining the character and properties of the micelle.

General Comment

The multiplicity of factors concerned make it difficult to define the physicochemical status of the different electrolytes in the serum with precision. Calcium has been extensively studied and it is now recognized that both the absolute amount and the proportion of calcium existing in the serum, in ionized or non-ionized forms, are dependent on the concentration of calcium, as well as that of protein, and are affected not only by changes in the carbon dioxide tension and hydrogen ion concentration, but also by the concentration of other ions, as chlorides and phosphates (3, 9, 16, 21).

The effect of changes in the hydrogen ion concentration and carbon dioxide tension in the calcium, as pointed out by Greenberg and Gunther (9), ordinarily is so slight as to be negligible, but this is not true with regard to other ions. Our own experiments with ultrafiltration and with dialysis *in vitro* have indicated that very considerable shift in the sodium and chloride ratios may occur between pH 6.6 and 8.2, and that the loss of carbon dioxide in the usual apparatus for ultrafiltration is sufficient to vitiate the value of the results for studies such as these.

The evidence for the effect of changes in the concentration of one ion in the serum on the behavior of another, such as the effect of changes in the concentration of chlorides or phosphates on the behavior of the calcium, suggests that the Debye-Hückel theorem, which takes the dielectric constant of the solution into consideration, may have an application in explaining the changes observed. This theorem has been applied by Stadie (23) to the study of the

interaction of hemoglobin, bicarbonate-sodium chloride systems. Unfortunately too little is known of the necessary constants to permit its application to the study of the state of the different electrolytes in the blood serum, at the present time.

SUMMARY

A study has been made of the distribution of electrolytes across a collodion membrane between blood serum and a dialysate obtained *in vivo*.

The chloride and bicarbonates in the serum apparently exist in the free state. The inorganic phosphates, on the other hand, are partially held in a non-diffusible form.

The different bases in the serum exist in part in combination with the serum protein. The ratio between the cations combined with diffusible anions in the serum and dialysate was assumed to be the same as the ratio of the chlorides in the two solutions. The excess of bases in the serum over the amount calculated in this way was assumed to represent the non-diffusible fraction combined with protein. The results indicate that the different bases are bound to the protein in a non-diffusible form to the extent of approximately 11 per cent of the sodium, 24 per cent of the potassium, and between 35 and 45 per cent of the calcium and magnesium. The amount of base bound by the serum proteins varied from 2.48 to 3.93 with an average value of 3.16 milli-equivalents for each per cent of protein. Reasons are given for considering that this base proteinate is only partially ionized.

The present knowledge of the physicochemical laws which govern the behavior of colloidal solutions is not sufficient to permit the complete characterization of such a system as blood serum. This study of the electrolyte equilibrium between the blood serum and the dialysate has amplified, and in general has confirmed, the results of previous experiments *in vitro*. Various theories of colloidal behavior apply in part to the study of the above system, but the characteristic behavior of the different ions necessitates appropriate modification in the application of each theory.

The comparison of these results with those obtained in the study of other body fluids, such as transudates, or the cerebrospinal fluid, will be reported later.

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THE DISTRIBUTION OF ELECTROLYTES BETWEEN SERUM AND TRANSUDATES

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That there is a disturbance in water metabolism in cases of edema or ascites is obvious. Many investigations have shown that there is a simultaneous disturbance in the metabolism of the inorganic salts. In consequence, the cause of the edema has been sought in the chemical composition of the pathologic fluid or in the physicochemical relationship between the composition of the fluid and the blood serum.

Previous analyses of the blood serum and transudates in human subjects have indicated a distribution of electrolytes between the two solutions similar to that obtained after the equilibration *in vitro* of horse serum and artificial salt solutions across a collodion membrane. These results have been quoted widely by the proponents of a physicochemical theory of edema formation. Unfortunately only a relatively small number of such analyses have been made and the results of experiments with serum from different species of animals have been compared directly. In the present report we wish to present the results of a considerable series of analyses of serum and ascitic fluid in dogs and in clinical cases. The distributions obtained have been compared with those which occur between the serum and the *in vivo* dialysate previously reported by Greene and Power (5).

Electrolyte Distribution in Dogs

Experimentally produced edema or ascites, in a dog, ordinarily is transitory. The spontaneous development of ascites, however, has been observed in dogs after experimental ligation of the com-

mon bile duct (12), and Bollman (3) has shown in these animals the intimate relation between the diet and the appearance of the peritoneal effusion. Because the ascites can be maintained indefinitely under these conditions, such animals afford an opportunity for the study in dogs of the distribution of electrolytes between serum and ascitic fluid, and for the comparison of the results, both with the data obtained in clinical cases and with the *in vivo* dialysate of the blood obtained experimentally in dogs.

Material and Methods—The common ducts of ten dogs were doubly ligated and sectioned aseptically, ether anesthesia being used. 1 to 2 months later, ascites was produced by the feeding of considerable quantities of meat or meat extract, as described by Bollman (3). Samples of the ascitic fluid, obtained by paracentesis, were collected over mercury without exposure to the air. Samples of blood were drawn from the jugular vein without stasis. They were collected under oil and centrifuged immediately so that the corpuscles were thrown down before coagulation occurred. They were then put on ice until coagulation was complete, when they were recentrifuged and the serum was removed. The analytic methods were those previously described (5).

Results—The results of these analyses in dogs are given in Table I, Series 1. The protein content of the serum in these dogs was slightly reduced below that seen in normal animals. The ascitic fluid on the other hand contained very small amounts of protein. Compared to the serum, the protein, calcium, magnesium, and phosphates in the ascitic fluid are reduced. The sodium and potassium are essentially the same as the serum and the chloride and carbon dioxide in the ascitic fluid are greater than the serum. The values obtained for the different inorganic constituents of serum and for the distribution between the serum and the transudate agree well with those previously reported by Loeb, Atchley, and Palmer (9), as well as by Hastings, Salvesen, Sendroy, and Van Slyke (7), and by Gollwitzer-Meier (4).

Electrolyte Distribution in Clinical Cases

Material and Methods—Samples of ascitic or pleural fluid were obtained by paracentesis from ten patients. Samples of blood were drawn from the median cubital vein without stasis. All samples were collected under oil, and were handled rapidly and

with care to prevent loss of carbon dioxide. Some of the patients had been treated by the administration of ammonium nitrate; the nitrates present in the serum and fluid were determined by the method of Whelan (15).

Results—The analytic results are given in Table I, Series 2. The protein content of the serum was essentially normal in these cases although it was reduced to 3.7 per cent in one case. The ascitic and pleural fluids contained considerably more protein than that found in the animal experiments. Clinical acidosis was not present in this group of cases. The amount of nitrates present in the serum depended on the previous medication received by the patient. In those cases in which ammonium nitrate was administered, the nitrates in the serum varied from 0.10 to 5.55 mg. for each 100 cc., with an average value of 3.51 mg. The fluid usually contained somewhat more, the values ranging from 0.17 to 6.95 mg.

The distribution of the different ions between the serum and the pleural and ascitic fluids in these cases in general is the same as that observed in the dogs. Compared to the serum, the protein, potassium, calcium, and phosphates in the transudate are reduced; the sodium and magnesium are essentially the same as in the serum, whereas the chlorides and nitrates in the transudate are greater than in the serum.

General Comment

A study of the equilibrium between the serum and the transudate is best made on the basis of the data given in Tables II and III. In Table II, the various constituents are expressed as milliequivalents for each kilo of water in the serum or transudate. The amount of water was determined directly in all cases. The equivalent bicarbonate was calculated on the assumption that one-twenty-first of the carbon dioxide content represented free carbonic acid. This method of calculation does not take the variation in hydrogen ion concentration into account, but there was very little difference in each case between the hydrogen ion concentration of the serum and that of the transudate, so that this omission does not introduce any significant error. The concentration of inorganic phosphate was converted into its equivalent base-binding power at pH 7.4 (1.8 times the millimolal concentration of phosphate).

TABLE I

Composition of Blood Serum and Transudates

Na, K, Ca, Mg, chloride, phosphate, and nitrate N are measured in mg. per 100 cc.

Series 1 Dog No.	Serum water	Fluid water	Serum protein	Fluid protein	Na		K		Ca		Mg		Cl(NaCl)		PO₄ (P)		CO₂, per cent by volume		Nitrate N	
					Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 gm.	gm. per 100 gm.																
1	95.24	99.27	5.40	0.31	335	332	18.9	20.2	9.26	6.42	2.70	2.10	683	733	4.05	4.70	50.5	58.8		
2	94.20	99.03	5.03	0.40	329	337	17.3		8.78	7.04	2.4	2.30	673	731	3.88	3.60	38.2	48.9		
3	94.76	98.89	5.20	0.17	350	345	17.6	15.3	10.76	8.20	2.59	1.70	638	687	4.64	5.03	57.6	71.1		
4	94.53	98.92	5.20	0.34	350	349	18.5	18.6	9.70	6.91	2.69	2.13	691	746	3.86	4.49	49.9	51.3		
5	95.06	98.64	4.50	0.35	369	353	23.0	23.4	8.28	6.40	2.70	2.0	759	793	7.31	5.15				
6	94.18	98.81	6.20	0.27	356	350	19.5	21.9	8.56	6.68	1.56	2.70	684	734	3.70	3.94	55.1	59.5		
7	95.12	99.27	4.55	0.10	347	346	19.9	16.5	9.16	6.64	1.85	1.85	645	700	4.12	4.60	61.0	69.1		
8	95.24	99.12	5.22	0.24	345	328	19.5	20.0	9.60	6.62	2.41	2.06	695	739	4.12	4.10	58.8	64.9		
9	93.10	98.78	7.09	0.65	333	330	22.6	19.1	9.51	6.70	3.25	2.02	646	708	4.49	3.53	51.8	61.8		
10	93.56	98.25	7.10	0.75	339	331	19.0	20.1	9.53	7.29	2.69	1.81	660	722	4.88	4.26	51.8	57.5		
Average..	94.50	98.90	5.54	0.36	345	340	19.5	19.8	9.31	6.89	2.48	2.07	677	729	4.50	4.34	52.7	60.4		
Series 2 Patient No.																				
1*	93.02	97.12	7.24	3.10	307	311	13.1	12.6	10.04	7.63	2.39	2.09	518	566	4.49	4.68	71.3	71.0	0.42	0.59
2†	93.81	95.46	6.15	4.57	320	320	18.3	14.8	9.64	8.58	3.09	2.80	600	623	4.47	3.74	61.0	58.3	1.22	2.00
3‡	94.00	95.22	6.76	5.00	335	333	18.6	12.5	10.18	9.35	1.31	2.35	577	590	4.19	4.00	61.9	60.3	2.80	2.80
4§	93.44	95.99	7.38	3.82	316	318	15.5	13.7	9.40	7.97	2.21	2.05	555	571	2.89	2.93	69.5	71.5	3.70	4.80

5§	93.30	95.92	7.22	4.01	333	319	15.0	14.8	9.42	7.94	2.24	1.99	561	586	3.18	3.02	66.2	63.8	3.78	4.45
6‖	95.11	99.02	3.52	1.95	305	301	20.2	12.5	7.52	5.24	2.17	1.92	609	651	2.40	2.34	50.2	51.3	0.10	0.17
7¶	93.48	97.80	6.73	2.12	308	331	15.7	12.6	8.11	6.62	3.1	2.6	560	640	2.38	3.10	62.2	66.7	5.55	6.95
8¶	93.23	95.98	6.67	3.34	315	317	17.2	14.0	9.10	7.80	1.94	1.97	542	573	4.02	3.76	64.0	63.6		
9†	93.84	96.65	4.99	2.49	361	332	20.0	12.0	9.70	7.87	2.56	2.58	582	620	3.90	4.00	60.1	55.9		
10¶	93.41	98.28	5.80	0.56	333	320	17.4	10.8	11.08	7.82	2.55	2.36	508	544	3.27	3.02	79.5	85.8		
Average..	93.66	96.74	6.25	3.09	323	320	17.1	13.0	9.42	7.68	2.36	2.27	561	599	3.52	3.46	64.6	64.8	2.51	3.11

* Hydrothorax; cardiac.
† Carcinomatous ascites.
‡ Carcinomatous hydrothorax.
§ Ascites; cardiac failure.
‖ Hydrothorax; nephrosis.
¶ Ascites; portal cirrhosis.

TABLE II—*Electrolyte Equilibrium*

Values are expressed in milli-equivalents per kilo of water.

Series 1 Dog No.	Serum water	Fluid water	Serum protein	Fluid protein	Na		K		Ca		Mg	
					Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid
	gm. per 100 cc.	gm. per 100 cc.	gm. per 100 gm.	gm. per 100 gm.								
1	95.24	99.27	5.67	0.31	153.0	145.3	5.1	5.2	4.9	3.2	2.3	1.7
2	94.20	99.03	5.34	0.40	151.7	147.8	4.7		4.7	3.6	2.1	1.9
3	94.76	98.89	5.49	0.17	160.4	151.7	4.8	4.0	5.7	4.1	2.2	1.4
4	94.53	98.92	5.50	0.34	160.9	153.5	5.0	4.8	5.1	3.5	2.3	1.8
5	95.06	98.64	4.73	0.35	168.7	155.6	6.2	6.1	4.4	3.2	2.3	1.7
6	94.18	98.81	6.58	0.27	164.3	154.0	5.3	5.6	4.5	3.4	1.4	2.2
7	95.12	99.27	4.78	0.10	158.7	151.7	5.3	4.2	4.8	3.3	1.6	1.5
8	95.24	99.12	5.48	0.24	157.4	143.9	5.2	5.2	5.0	3.3	2.1	1.7
9	93.10	98.78	7.61	0.66	155.6	147.4	6.2	5.0	5.1	3.4	2.9	1.7
10	93.56	98.25	7.60	0.76	157.8	146.5	5.2	5.2	5.1	3.7	2.4	1.5
Average..	94.50	98.90	5.88	0.36	158.9	149.7	5.3	5.0	4.9	3.5	2.2	1.7
Series 2 Patient No.												
1	93.02	97.12	7.78	3.19	143.5	139.0	3.6	3.3	5.4	3.9	2.1	2.0
2	93.81	95.46	6.56	4.79	148.2	145.6	5.0	4.0	5.1	4.5	2.7	2.4
3	94.00	95.22	7.19	5.25	155.2	152.2	5.0	3.3	5.4	4.9	1.2	2.0
4	93.44	95.99	7.90	3.98	147.0	144.7	4.2	3.7	5.0	4.2	1.9	1.8
5	93.30	95.92	7.74	4.18	155.2	144.7	4.1	3.9	5.1	4.1	2.0	1.7
6	95.11	99.02	3.70	1.97	139.5	132.2	5.4	3.2	4.0	2.6	1.9	1.6
7	93.48	97.80	7.20	2.17	143.0	147.3	4.3	3.3	4.3	3.4	2.7	2.2
8	93.23	95.98	7.16	3.48	147.0	143.4	4.7	3.7	4.9	4.1	1.7	1.7
9	93.84	96.65	5.32	2.58	167.3	149.5	5.5	3.2	5.2	4.1	2.2	2.2
10	93.41	98.28	6.21	0.57	155.2	141.7	4.8	2.8	5.9	4.0	2.2	2.0
Average..	93.66	96.74	6.68	3.22	150.1	144.0	4.7	3.4	5.0	4.0	2.1	2.0

between Serum and Transudate

Cl		Bicarbonate		P		Nitrate		Total cations		Total anions		Cation excess	
Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid
122.6	125.8	22.7	25.5	2.5	2.8			165.3	155.4	147.8	154.1	17.5	1.3
122.0	126.2	17.3	21.0	2.4	2.1			163.2	158.0	141.7	149.3	21.5	8.7
115.0	118.8	26.0	30.8	2.8	3.0			173.1	161.2	143.8	152.6	29.3	8.6
125.0	128.8	22.6	22.1	2.4	2.6			173.3	163.6	150.0	153.5	23.3	10.1
136.4	137.4	24.0	26.1	4.5	3.0			181.6	166.6	164.9	166.5	16.7	0.1
124.2	127.0	25.0	25.8	2.3	2.3			175.5	165.2	151.5	155.1	24.0	10.1
115.9	120.5	27.4	29.8	2.5	2.7			170.4	160.7	145.8	153.0	24.6	7.7
124.9	127.5	26.4	28.0	2.5	2.4			169.7	154.1	153.8	157.9	15.9	3.8
118.7	122.6	24.8	26.8	2.8	2.1			169.8	157.5	146.3	151.5	23.5	6.0
120.5	125.6	23.7	25.0	3.0	2.5			164.5	156.9	146.3	153.1	17.3	3.8
122.5	126.0	24.0	26.1	2.8	2.5			171.3	159.9	149.3	154.6	22.0	5.3
99.3	99.6	32.8	31.3	2.8	2.8	0.32	0.44	154.6	148.2	131.2	134.1	23.4	14.1
109.4	111.6	27.7	26.1	2.8	2.3	0.93	1.50	161.0	156.5	140.8	141.5	20.2	15.0
109.0	106.0	28.2	27.1	2.6	2.4	2.13	2.10	166.8	162.4	137.9	137.6	28.9	24.8
109.5	102.2	31.8	31.9	1.8	1.8	2.83	3.59	158.1	154.4	137.9	139.5	20.2	14.9
108.8	104.4	30.4	28.5	2.0	1.8	2.90	3.32	166.4	154.4	138.1	138.0	28.3	16.4
109.4	112.3	22.6	22.2	1.5	1.4			150.8	139.6	133.5	135.9	17.3	3.7
109.4	111.7	28.6	29.1	1.5	1.8	0.79	1.24	154.3	156.2	133.3	143.8	21.0	12.4
99.4	102.0	29.3	28.4	2.5	2.3	4.25	5.18	158.3	152.9	135.4	138.0	22.9	14.9
106.0	109.6	27.4	24.8	2.4	2.4	.		180.2	159.0	135.8	136.8	44.4	22.2
99.0	98.3	36.5	37.3	2.0	1.8			168.1	150.5	131.5	137.4	36.6	13.1
108.4	105.8	29.5	28.7	2.2	2.1	1.92	2.32	161.9	153.4	136.0	138.8	25.9	14.6

When the composition of the serum and transudate are compared on the basis of the data in Table II it is apparent that the concentrations of the various bases are uniformly higher in the

TABLE III

Ratios between the Electrolyte Concentration of Serum and Transudate

Series 1 Dog No.	$\frac{\text{Na in fluid}}{\text{Na in serum}}$	$\frac{\text{K in fluid}}{\text{K in serum}}$	$\frac{\text{Ca in fluid}}{\text{Ca in serum}}$	$\frac{\text{Mg in fluid}}{\text{Mg in serum}}$	$\frac{\text{Cations in fluid}}{\text{Cations in serum}}$	$\frac{\text{Chloride in serum}}{\text{Chloride in fluid}}$	$\frac{\text{CO}_2 \text{ in serum}}{\text{CO}_2 \text{ in fluid}}$	$\frac{\text{P in serum}}{\text{P in fluid}}$	$\frac{\text{Nitrate in serum}}{\text{Nitrate in fluid}}$	$\frac{\text{Anions in serum}}{\text{Anions in fluid}}$
1	0.949	1.020	0.644	0.743	0.940	0.974	0.890	0.847		0.960
2	0.974		0.767	0.910	0.968	0.968	0.824	1.132		0.950
3	0.946	0.834	0.730	0.630	0.931	1.050	0.844	0.963		0.942
4	0.954	0.961	0.682	0.754	0.944	0.970	1.023	0.899		0.977
5	0.923	0.984	0.744	0.715	0.917	0.993		1.472		0.991
6	0.937	1.067	0.744	1.653	0.942	0.977	0.969	0.985		0.977
7	0.956	0.794	0.694	0.954	0.944	0.962	0.919	0.934		0.953
8	0.914	0.984	0.662	0.822	0.908	0.979	0.943	1.045		0.974
9	0.934	0.798	0.664	0.588	0.927	0.968	0.925	1.346		0.966
10	0.929	1.013	0.728	0.641	0.954	0.959	0.948	1.200		0.961
Average..	0.943	0.944	0.715	0.773	0.934	0.972	0.920	1.120		0.965
Series 2 Patient No.										
1	0.969	0.917	0.722	0.952	0.958	0.957	1.048	1.000	0.740	1.979
2	0.976	0.800	0.883	0.889	0.972	0.980	1.061	1.218	0.620	0.996
3	0.981	0.660	0.908	1.666	0.974	0.991	1.040	1.083	1.010	1.003
4	0.985	0.881	0.840	0.948	0.976	0.994	0.997	1.000	0.790	0.989
5	0.933	0.951	0.804	0.850	0.928	0.986	1.066	1.111	0.870	1.000
6	0.947	0.593	0.650	0.842	0.926	0.974	1.018	1.071		0.983
7	1.030	0.768	0.791	0.815	1.012	0.917	0.983	0.834	0.640	0.926
8	0.978	0.787	0.837	1.000	0.966	0.975	1.032	1.087	0.820	0.982
9	0.894	0.582	0.788	1.000	0.882	0.967	1.104	1.000		0.993
10	0.913	0.584	0.678	0.910	0.895	0.946	0.979	1.112		0.957
Average..	0.961	0.752	0.790	0.987	0.949	0.969	1.033	1.052	0.830	0.9798

serum, whereas the concentrations of the various anions tend to be higher in the transudates. These ratios are given directly in Table III. In dogs the average base ratios range from 0.942 for sodium to 0.71 for calcium, whereas in clinical cases the ratios are

slightly higher, 0.961 for sodium and 0.79 for calcium. In both instances the base ratios are higher than those obtained by dialysis *in vivo*. This is to be expected as the difference between the protein content of the two solutions is less in the case of these transudates than it was in the case of the dialysates.

The distribution of the various anions was somewhat less regular than that of the various bases. In both dogs and man the phosphates were higher in the serum than they were in the transudate. This result, again, is in harmony with the results of dialysis *in vivo*, which indicated that some of the inorganic phosphate in the serum was held in non-diffusible combination. The chlorides uniformly were greater in the transudates than in the serum and were of approximately the same magnitude as those obtained by dialysis *in vivo*. The bicarbonate ratio in the experiments with dogs was approximately the same as that of the chlorides but in man the transudate contained less bicarbonates than the serum. Loeb, Atchley, and Palmer (9) found the same relationships to be present in their comparison of transudates with blood from the arm. One of the difficulties in studies such as this is that of obtaining a sample of blood truly representative of that with which the particular transudate in question is in equilibrium. The true equilibrium in the case of ascitic fluid may be either with the blood from the mesenteric artery, the portal vein, or the intervening capillaries. In either case, blood from the median ˙cubital vein at best could indicate only the approximate state of the equilibrium present. Such considerations probably will serve to explain the apparently anomalous bicarbonate ratio present in these cases.

Seven of the patients had been taking ammonium nitrate. The concentration of nitrate in the serum and in the ascitic fluid of one patient was the same, but there was a great excess of nitrate in the ascitic fluid of the other six patients, so that the average serum-fluid ratio was 0.83 (this may be due to the time at which samples were taken, for it is possible that the nitrate content of the ascitic fluid tends to lag behind the serum, both when the amount in the serum is increasing during the administration of ammonium nitrate and when the amount in the serum is decreasing after administration of this drug has been stopped). Hastings and van Dyke (6) have found that after sodium bromide has been

given there is an irregular distribution of bromides which concentrate in the erythrocytes at the expense of the chlorides, but that if the sum of the two anions is considered the expected distribution ratios between cells and serum are found. The same may be true of the different anions present in these transudates.

In a consideration of the dialysis of the blood, *in vivo*, the distribution of the chlorides was assumed to represent the best measure of the shift in concentration produced by the membrane equilibrium present, and the chloride ratio was used for calculating the state of the various cations present in the serum. It was further shown for the purposes of this discussion that the serum could be considered as a diphasic system, consisting of colloidal micelles dispersed in the intermicellary fluid or free dispersion medium present. The collodion membrane theoretically serves to separate the two phases without altering the equilibrium already established between micelles and fluid. In consequence the dialysate may be considered to represent the composition of this intermicellary fluid.

The same general considerations apply to the relationship between the composition of the serum and transudate. Both solutions contain protein but in varying amounts. The membrane separating the serum is assumed to be freely permeable to water and to all ions except the protein anions. To maintain osmotic equality the ratio of osmotically active molecules and ions to water must be the same in both serum and transudate.

$$A_s + B_s = A_f + B_f \tag{1}$$

Complete dissociation of the electrolytes being assumed, the cations in either fluid may be considered as the sum of the cations paired with diffusible anions and those bound by protein. We then have

$$2A_s + BP_s = 2A_f + BP_f \tag{2}$$

The concentration of the different electrolytes in serum and transudate is so nearly the same that these relationships still hold, even if we assume not complete dissociation but equal dissociation of the various salts. Dividing the equation through by $2A_f$ and rearranging, we find that

$$r_{sf} = \frac{A_s}{A_f} = 1 - \frac{BP_s - BP_f}{2A_f} \tag{3}$$

According to this calculation the protein in the fluid may be considered as the equivalent of a diffusible anion which neutralizes the effect of a corresponding amount of protein in the serum. If one assumes that the protein in the two solutions is ionized to a like extent, then the distribution ratio between the total determined inorganic anions in the serum and transudate is a measure of the Donnan effect produced by the excess of protein in the serum. Accordingly the state of the various bases present in the serum may be calculated from the composition of the serum and the transudate, in the same manner as previously was done in the study of the serum and the *in vivo* dialysate. The average base-binding power of the serum proteins, when so calculated from the data given in the present series of analyses, compares favorably

TABLE IV

Base Bound by Serum Protein

Experiment series	M.-eq. per 1 per cent protein					Bound water per 1 per cent protein	Ionization of base protein-ate
	Na	K	Ca	Mg	Total base		
						cc.	per cent
Dialysate..................	2.55	0.166	0.355	0.10	3.16	0.276	27
Ascitic fluid, dogs...........	2.60	0.085	0.297	0.11	3.07	0.633	67
Transudates, men...........	2.55	0.388	0.329	0.06	3.29	0.567	53
Average..................	2.57	0.21	0.327	0.09	3.17	0.482	49

with that previously obtained. The average results for each gm. of protein are shown in Table IV. The values obtained were all of approximately the same magnitude in the three series of experiments and there was very good agreement between the figures for the total base and the sodium combined with protein. The results for calcium and magnesium were less uniform and the greatest variation was present in the figures for potassium. These differences, however, are no greater than is to be expected from a consideration of the analytic errors in each case. We have previously considered the difficulties in the use of a figure such as this for the base-binding power of the serum proteins. Nevertheless for the present, the average values given in Table IV may be

regarded as the most satisfactory figures available for the calculation of the base-binding power of the serum proteins.

It was pointed out (5) that the anions in the serum, with the exception of the phosphates, probably all occur in the free state. The results of the present series of analyses accord with this view, with the sole exception of the bicarbonate ratios in the transudates from man. We have already considered this point and have given our reasons for thinking that this ratio does not represent the actual bicarbonate equilibrium. The concentration of anions in the dialysate or transudate is increased over that in the serum by virtue of the Donnan equilibrium that is present between the two solutions.

The data in Table IV indicate that the amount of the different bases bound to protein apparently is a constant, and is independent of the type of membrane, whether it is a collodion or a living membrane, that is responsible for the equilibrium from which the base-binding power of the serum proteins is calculated. The apparent degree of ionization of the base proteinate varies considerably in the three series of experiments as does the amount of water held in the protein micelle. Both these constants represent calculated values which were not determined directly, and small experimental errors will produce considerable variation in this calculation. A collodion membrane immersed in serum usually carries a negative electrical charge and it is recognized that variations in this charge will affect the electrolyte equilibrium across a dialyzing membrane. The charge on the capillary wall or on other·living animal membranes is not known and a comparison of the effect of this charge with that on a collodion dialyzing membrane is not possible. Differences in the electrical charge of the membranes, however, may well affect the apparent degree of ionization of the serum protein when calculated by the method used here.

The dialysate may be used as an index to the composition of the intermicellary fluid or free dispersion medium present in the serum. On the basis of this assumption the apparent difference between the composition of the two solutions may be explained by some of the water present in the serum being held within the zone of electrostatic attraction of the protein micelle. The amount of water so held for each gm. of protein is shown in Table IV. The average is 0.5 cc. Depending on whether the specific volume of 1

gm. of serum protein is taken as 0.75 (13) or 0.80 (14) this gives a value of 1.25 or 1.30 as the specific volume of the protein micelle. These values agree well with those of 1.2 and 1.3 previously reported by Polányi (11) and Augsberger (2). Similar values have been obtained by Moran (10), and by Adair and Callow (1), in the study of gelatin gels. Hill (8) also has found that 2 to 3 per cent of the water in blood and other protein solutions is bound by the colloid.

In a previous study (5) it was pointed out that certain modifications in the application of the currently accepted physicochemical laws relative to the properties of protein solutions were necessary to explain the relationship observed between blood serum and the dialysate *in vivo*. If these modifications are kept in mind, then the results reported here confirm the earlier studies of Loeb, Atchley, and Palmer (9), of Hastings, Salvesen, Sendroy, and Van Slyke (7), and of Gollwitzer-Meier (4) in indicating that the distribution of the various electrolytes between blood serum and transudate is governed by the same physicochemical laws which determine the distribution of these ions between serum and its dialysate across a collodion membrane. In consequence, the relative amounts of protein present in the two solutions determine the relative distribution of the various ions between the serum and transudate. The fact that the distribution across the living membrane, whether this is capillary wall or pleural or peritoneal serosa, or the combination of the two, is the same as that across a collodion membrane is perhaps without direct biologic significance. Although physicochemical laws will determine the composition of the transudate while it is in equilibrium with the blood serum they do not explain either the development or the resorption of the transudate. Changes in the permeability of the living membrane must be assumed to explain the varying amounts of protein in the different transudates. Many conflicting hypotheses have been advanced to explain the causation of edema and ascites and a review of this question is beyond the scope of this report. We wish rather to point out that the explanation is not to be found in the composition of the transudate alone for at equilibrium this is determined by physicochemical laws.

SUMMARY

A study has been made of the distribution of electrolytes between blood serum and experimentally induced transudates (ascitic fluid) in dogs and pathologically occurring transudates in clinical cases. There was no physiologically significant difference between the two series of experiments.

The distribution of the various electrolytes between blood serum and transudates was apparently governed by the same physicochemical laws which determined the distribution of these same ions between the serum and its *in vivo* dialysate. Under these conditions, the relative amount and character of the protein present in the two solutions seemingly is the factor which primarily determines the relative distribution of the various ions between the serum and transudate.

The base-binding power of the serum protein calculated from the electrolyte equilibrium between the serum and transudates is approximately the same as that calculated from the composition of the *in vivo* dialysate.

It is pointed out that although these physicochemical laws will determine the composition of a transudate while it is in equilibrium with the blood stream they do not explain either the development or the resorption of the transudate.

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A METHOD FOR THE DETERMINATION OF THE FREEZING POINT DEPRESSION OF AQUEOUS SOLUTIONS PARTICULARLY THOSE CONTAINING PROTEIN

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Two different principles are used in current freezing point methods.

1. The equilibrium vessel is immerséd in an air bath at a temperature 2–3° below the freezing point, the solution therein is thereby supercooled, a part of the solvent is frozen out, initiated usually by seeding and equilibrium between solid and solvent, and solution is established by stirring. The supercooling and the increase of concentration on freezing out of solvent tend to give unduly high depressions. The ordinary Beckman method is an example.

2. The solution of approximate concentration is mixed with small pieces of pure ice and stirred efficiently by a mechanical stirrer or pump. When equilibrium is reached a sample of the solution is quickly taken from the apparatus and its exact composition determined by analysis or it may be analyzed *in situ* by conductivity measurements. The equilibrium vessel is immersed in a bath at 0° or close to the freezing point of the solution and thus supercooling is avoided. Since some ice is melted in bringing the solution, ice, and apparatus to the equilibrium temperature, dilution of the original solution (10 to 30 per cent) occurs. Hence, this method cannot be used to determine the freezing point of any given solution such as serum but serves excellently to plot a curve of freezing points against concentration of one solute at varying concentrations, and is the method used in the more recent accurate determinations of freezing points in dilute solutions (Havorka and Rodebush, 1925).

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Two temperature reading devices are used and these determine the accuracy with which the freezing point may be read. The first is the Beckman thermometer reading to 0.001°, the second is the thermocouple which with precise technique is susceptible of an accuracy of 0.00002° (Havorka and Rodebush, 1925).

The method reported here differs in principle from those outlined above but resembles the second more than the first. The solution in a cooling vessel is cooled to a temperature within less than 0.1° of its freezing point which as a rule is approximately known or, as later detailed, approximated in the course of the determination. It is then transferred to a second (equilibrium) vessel, which contains several small pieces of ice which (as well as the equilibrium vessel, thermometer, and stirrer) have also been precooled to the approximate freezing temperature and the final exact equilibrum is rapidly attained. The precooling of the solution, ice, and equilibrium vessel and the fact that the latent heat of fusion of ice is high, minimizes the melting of ice necessary to attain equilibrium so that the dilution of the original solution when it is transferred to the equilibrium vessel is negligible for most purposes. The whole apparatus is immersed in a bath adjusted by the addition of brine to within less than 0.1° of the freezing point so that supercooling is impossible. The disadvantages of the first two methods are overcome with no increase in technical difficulties and within the limits for which the method was designed, employing a Heidenhain thermometer; we estimate conservatively that the method is accurate to within ±0.001° equivalent to ±0.6 mM of osmolar concentration.

We have employed the method in a large series of determinations of freezing points of solutions of hemoglobin of varying composition and of sera of pneumonia patients and we believe it to be eminently satisfactory for protein-containing solutions though of course it may be used for simple aqueous solution not too dilute. It should also be borne in mind that when we are comparing the freezing points of sera in normal and pathological conditions where the variation may be less than 0.1° the maximum error may be a large fraction of the variation.

Apparatus

The details of the apparatus are shown in Fig. 1. On the left is the *equilibrivm vessel* which consists of a tube 15 × 30 mm. sur-
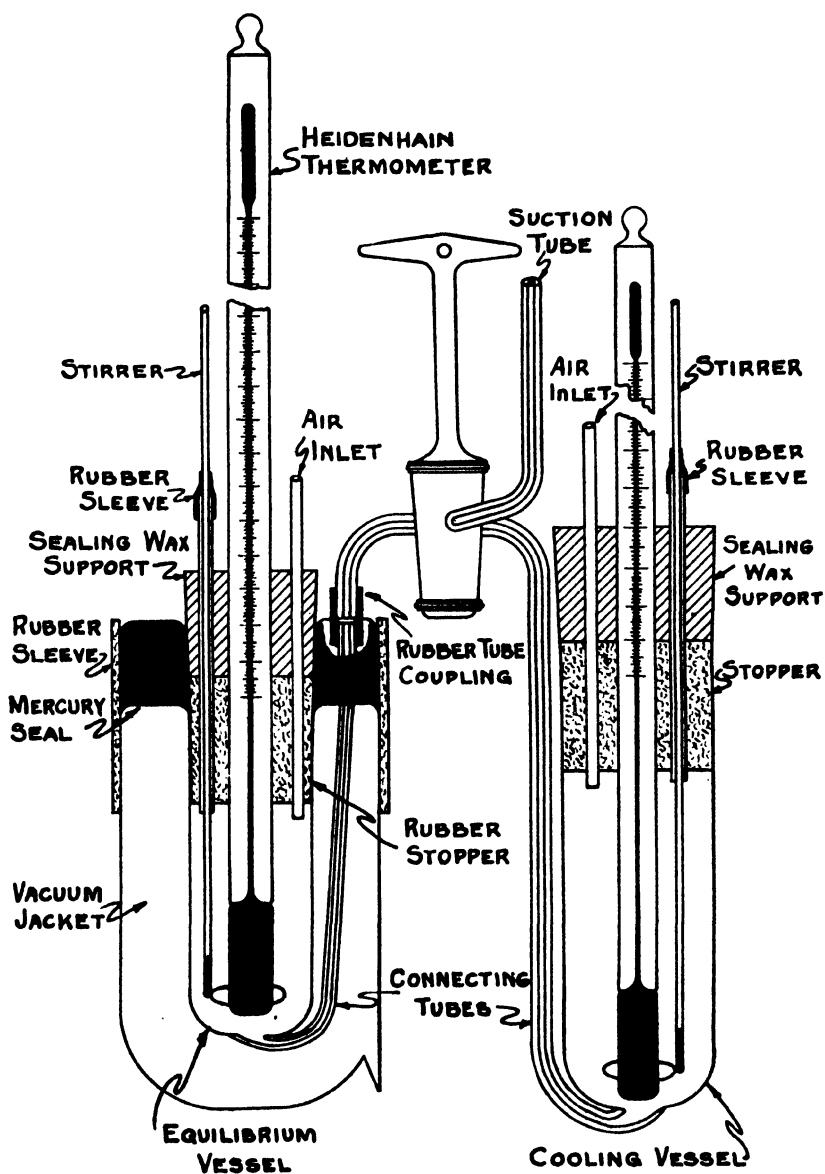
FIG. 1. Freezing point apparatus

rounded by a *vacuum jacket*. A rubber stopper holds the Heidenhain *thermometer*, a glass tube to guide the *stirrer*, and a second smaller glass tube to act as an *air inlet*. When the stirrer is in place a small bit of rubber tubing fits snugly over it and its holder and acts as a rubber sleeve to prevent condensed moisture from the air from running into the equilibrium vessel. The thermometer, stirrer, and inlet tube, are rigidly supported in the rubber stopper by an extension of *sealing wax*, which is made by pouring melted sealing wax into a paper cylinder mold wound around the stopper. A bit of wide rubber tubing about the top of the equilibrium vessel forms the outside support for a mercury seal. This seal and the rigid setting of the thermometer effectually prevent leakage from the brine bath in which the entire apparatus is immersed.

The bottom of the equilibrium vessel connects with a tube 2 mm. in bore which passes up and through the jacket and a rubber connection connects it to a 3-way stop-cock as shown in the sketch to the *cooling vessel* on the right which is the same size as the equilibrium vessel but has no vacuum jacket or mercury seal. The stopper here is likewise fitted with a *stirrer* and *air inlet* as well as a small Beckman type thermometer reading only to 0.01°. The two main units of assembled apparatus are held in place by two strong brass spring clamps supported by a ⅜ inch upright brass rod which also carries a bakelite base for inferior support. This assembly can be raised and lowered at will into a glass bath 7 × 8 × 7 inches which is pierced at one end for the shaft of a motor-driven stirrer the bearing of which is housed in a small stuffing box. The bath is filled with finely chopped ice and water which is vigorously stirred by the motor stirrer to attain 0° and to which brine is added to attain temperatures below zero.

Technique

Ice washed by distilled water is crushed, 15 to 20 pea size pieces selected and placed in the equilibrium vessel together with 10 to 15 cc. of water, sufficient to cover the bulb of the Heidenhain thermometer. The solution is placed in the cooling vessel. The two vessels are connected, placed in the stand, the mercury seal made, and the apparatus is placed in the bath which is filled with a mixture of ice and water rapidly stirred, to a depth sufficient to

cover the stoppers. After 30 to 45 minutes when the entire apparatus and its contents have attained 0°, the ice-water mixture in the equilibrium vessel is stirred and readings of the thermometer taken at intervals of 4 to 5 minutes until constancy of 0.001° is attained; three to four readings usually suffice. After the proper stem correction is made this reading gives 0° for the thermometer used. The bath temperature is now lowered to within 0.1° of the approximated freezing point by adding saturated brine, a few cc. at a time. This temperature in the bath may be maintained with ease and nicety by the occasional addition of brine. The solution in the cooling vessel is now cooled to its approximate freezing point.

The ice in the equilibrium vessel is now washed with the solution. Suction is applied to the 3-way connection, the water from the equilibrium vessel withdrawn, and the ice drained as completely as possible by continuing the suction for 15 to 30 seconds after the bulk of the water is withdrawn. The stop-cock is then turned so as to connect the equilibrium vessel with the cooling vessel and by means of gentle air pressure applied by mouth to the air inlet tube about 3 cc. of the solution in the cooling vessel are forced over into the equilibrium vessel. By moving the stirrer, the pieces of ice, thermometer bulb, and the lower part of the container are washed by the solution. This wash solution is now withdrawn by suction and the washing with 3 cc. samples of the solution repeated twice.

The washing of the ice being completed, 10 cc. of the solution are forced over into the equilibrium vessel. The outside bath temperature is adjusted and after gentle stirring of the equilibrium mixture the freezing point is read. A temperature constant to within 0.001° for 5 minutes is accepted as a final reading.

Efficiency of Washing Ice, Stirrer, etc.

We placed a 0.02 N acid solution in the equilibrium vessel together with ice and withdrew it in the manner described above. Titration showed that about 0.1 cc. of solution remained in the equilibrium vessel on the surface of the ice thermometer bulb, sides of the container, and stirrer. If three washings with 3 cc. of solution are routinely done only $\dfrac{0.1}{3^3}$ cc. of the water originally present in the equilibrium vessel remains. The dilution of the

10 cc. of the solution whose freezing point is being determined is then only $\frac{0.01}{3^s}$ or 1 part in 2700 less than 0.04 per cent.

Regulation of Outside Bath Temperature

Accurate freezing points are obtained only when the equilibrium vessel be immersed in a bath approximately at the freezing temperature. This precaution has likewise been emphasized by Jones and Bury (1927). Even a vacuum-jacketed equilibrium vessel does not prevent loss of heat from the solution. The following experiment (Table I) illustrates this point.

TABLE I

Influence of Bath Temperature on Freezing Point of Solution in Vacuum-Jacketed Equilibrium Vessel

Solution HCl Δ = 0.797°.

Temperature of bath	Excess bath temperature	Δ observed	Error in Δ
°C.	°C.	°C.	°C.
−0.76	0.04	−0.797	0.000
−0.84	0.04	−0.797	0.000
−0.94	0.14	−0.800	0.003
−1.04	0.24	−0.801	0.004
−1.14	0.34	−0.803	0.006
−1.24	0.44	−0.805	0.008
−1.90	1.10	−0.810	0.013
−1.90	1.10	−0.812	0.015
−1.90	1.10	−0.821	0.024
−1.86	1.06	−0.817	0.021
−1.80	1.00	−0.821	0.024

The last column shows that the bath temperatures more than 0.1° below the freezing point introduce errors. Experience with bath temperatures above the freezing point indicates the same limit for reliable results. The solution may be cooled to within 0.1° of its freezing point before final transfer to the equilibrium vessel or preferably before the first washing of the ice in two ways.

1. The freezing point is known to within 0.1°. After reading the zero of the thermometer, the bath temperature is rapidly lowered from 0° to the approximated freezing point and the solu-

tion cooled to this temperature. The washings of the ice are then made with the solution at a temperature not more than 0.1° from the freezing point. The contents of the equilibrium vessel are thus cooled so that when the final transfer of 10 cc. of solution is made the freezing point is closely approximated.

2. The freezing point of the solution is completely unknown. It may be approximated sufficiently during the washings. After the first washing at 0° the temperature in the equilibrium vessel will fall to some point intermediate between 0° and the freezing point. The bath and solution are lowered to this temperature and the second washing made. A further drop in the temperature occurs, the bath and solution are again lowered to this point, and the third washing is done. Another adjustment of the bath temperature is made before the final transfer of the solution is made for a reading. This series of approximations adjusts bath solution and equilibrium vessel to within 0.1° of the freezing point before the final transfer.

Duplicate Determinations

After the first determination, a second or third may be made by withdrawing the contents of the equilibrium vessel which if desired may be saved for further analysis, and transferring another 10 cc. sample from the cooling vessel to the equilibrium vessel. The duplicate determination is performed with rapidity since the entire apparatus, solution, ice, etc. are so close to the freezing point that equilibrium is attained within less than a minute.

Subsequent Determinations

When determinations on a number of solutions are made in one sitting, it is not necessary to start *de novo* for each solution. When the first determination is completed the apparatus is raised sufficiently in the bath to expose the rubber connection between the cooling vessel and the equilibrium vessel. The former is removed, cleaned, dried, and filled with the second solution, is then replaced, and the determination made as before. After determinations on a half dozen solutions it may be necessary to renew the ice in the equilibrium vessel.

Dilution of Solution

There are two ways in which the solution may become diluted in transfer from the cooling vessel to the equilibrium vessel: (1) dilution by the water which adheres to ice, stirrer, thermometer, and walls in the equilibrium vessel. We have already pointed out that this is of the order of 1 part in 2700. (2) It may become diluted by melting of ice in the attainment of final equilibrium. There are about 5 gm. of ice and 10 cc. of solution in the equilibrium vessel or 15 gm. of water whose temperature is to be lowered from some point between 0° and the freezing point. This requires 15 calories per 1° of lowering or, since the latent heat of fusion of ice is 80 calories, $\frac{15}{80}$ = 0.2 gm. of ice must be melted. If before the final transfer we adjust solution, ice, etc. to within 0.1° of the freezing point only 0.02 gm. of ice is melted which in the 10 cc. of solution gives a dilution of 2 parts in 1000 or 0.2 per cent. This error is smaller than the error due to the Beckman thermometer and practically in the field for which the method was designed we can say that there is negligible dilution of the solution after transfer. We have tested this using 0.02 N HCl and were unable by titration to demonstrate any dilution of the original solution when the procedures outlined had been followed and the solution withdrawn from the equilibrium vessel.

Sources of Error

1. Leakage of brine from the bath into the apparatus is a source of error. Well fitting stoppers with extensions of sealing wax described above to assure rigid support of thermometers and inlet tubes eliminate this. The danger of leakage is greater at the equilibrium vessel, since more stirring is done here and the rubber stopper supports a long heavy thermometer. The mercury seal and a clamp support on the thermometer after the apparatus is placed in the bath effectively eliminate the error.

2. A considerable amount of water condenses on the stirrer and may run into the vessel. The snug fitting pieces of rubber tubing fitted about stirrer and its support completely prevent this.

3. Large errors may be introduced by using alcohol and ether to dry any of the vessels with which the solution comes into con-

tact. Since both substances have a considerable effect on the freezing point of water, traces which inevitably cling to the sides of the vessels, despite careful and prolonged air-drying have in our experience caused large errors. We have dried all our apparatus including pipete by air suction only. Caprylic alcohol as an anti-foaming agent cannot be used as it has a considerable effect on the freezing point.

Results

To show the reproducibility of results on aqueous solution of purified salts we have made determinations on twenty to thirty different solutions (freezing point lowerings varied from 0.010° to 2.0°). Six determinations on each solution show in general the degree of reproducibility to be ±0.001°. Occasionally results differ by 0.003 to 0.005°.

In the comparison of the method with some other authoritative method we have used the data on the freezing point of solutions of KCl of Jones and Bury (1927) who brought to equilibrium in vacuum vessels large amounts of ice and solution and withdrew the solution at equilibrium for analysis.

These data are the best series of freezing point determinations on a purified salt in concentrated solution. The comparison not only gives the difference in the two methods of freezing point determination but also the differences in make-up and analysis of the solutions.

KCl was purified by three recrystallizations from distilled water and heated to fusion. Weighed amounts of salt were dissolved in weighed amounts of water and all dilutions were made by weight. Concentrations are expressed as mols per kilo of H_2O. The data of Jones and Bury up to 0.5 M may be represented by a straight line

$$\Delta = 3.284 \text{ M} + 0.018 \pm 0.0031$$

while our data from 0.1 to 0.6 M give

$$\Delta = 3.275 \text{ M} + 0.024 \pm 0.0041$$

where M = molality and Δ = freezing point lowering in °C. The difference of the two equations is

$$\Delta_{S \text{ and } S} - \Delta_{J \text{ and } B} = 0.006 - 0.009 \text{ M} \pm 0.0051$$

or at round concentrations   .

KCl *mols per kg.* *H₂O*	Difference of two methods °C.
0.1	0.005
0.2	0.004
0.3	0.003
0.4	0.002
0.5	0.001

In concentrated solution the two methods give identical results while in dilute solution the difference approximates the probable error of the two methods.

In more dilute solutions of NaCl and KCl, *viz.* those giving depression of 0.005 to 0.2°, we have obtained results differing in no case more than 0.003° from the best data in the literature (Adams, 1915). For less than 0.02° of depression the use of the Beckman thermometer excludes the possibility of precision.

SUMMARY

A new method for the determination of freezing point depression is described. The method avoids errors due to supercooling and changes in concentration of the solution. A Heidenhain thermometer is used for simplicity. In a depression greater than 0.020° an accuracy of ±0.001° is estimated.

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THE OSMOTIC COEFFICIENT OF SODIUM IN SODIUM HEMOGLOBINATE AND OF SODIUM CHLORIDE IN HEMOGLOBIN SOLUTION*

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INTRODUCTION

We report in this paper a continuation of the studies from this laboratory of the effect of protein (hemoglobin) on the thermodynamic properties of ions in solution. We have selected for that purpose a determination of the osmotic coefficient of ions as they coexist in aqueous protein solutions in their commonest modes; *viz.*, (1) Na^+ as alkali proteinate NaHb, (2) Na^+ and Cl^- as a neutral salt (NaCl) in a Hb solution. The osmotic coefficients were calculated from the freezing point depression as determined by the method of Stadie and Sunderman (1931).

Theory

The osmotic coefficient, φ, is by definition the ratio between observed (π) and theoretical (π_0) osmotic pressure or the corresponding freezing point depressions Δ and Δ_0. That is

$$\varphi = \frac{\pi}{\pi_0} = \frac{\Delta}{\Delta_0} = \frac{\Delta}{1.86\ (C)} \tag{1}$$

where (C) is the total concentration of all ions in mols per kilo of water and 1.86 is molal lowering of the freezing point of water.

In a solution of NaHb we have sodium ions and the Hb. The total depression is

$$\Delta = \Delta_{Na^+} + \Delta_{Hb}$$

Hb is present in small molecular concentration and we are able to make suitable correction for Δ_{Hb}. We make the assumption that changes in the osmotic activity coefficient of Hb when the concentration of Na is changed, if it occurs at all, will have a negligible effect upon the total Δ. We then get

$$\varphi_{Na} = \frac{\dot{\Delta}_{Na}}{1.86 \ (Na)} \tag{2}$$

Similarly for solutions of NaCl and NaHb we have

$$\Delta = \Delta_{NaCl} + \Delta_{NaHb}$$

Δ_{NaHb} is determined separately and by keeping NaHb small and making the assumption that its effect on Δ_{NaCl} (or vice versa) is negligible we get

$$\varphi_{NaCl} = \frac{\Delta_{NaCl}}{3.72 \ (NaCl)} \tag{3}$$

Method of Calculation of Concentrations—It is unimportant in dilute solution whether concentrations are calculated in mols per liter or mols per kilo of water. In concentrated solutions and in protein solutions on the contrary the two methods of calculation give quite different results. The question of choice between the two is important. We have not found any adequate discussion of the problem anywhere. Rivett (1911) in his studies on the osmotic pressure of salts in the presence of sugar recognized it but gives no solution. Most observers of osmotic pressure in concentrated solutions have calculated concentrations in mols per kilo of water. Debye and McAulay (1925), on the other hand, in their study of the osmotic pressure of salts in the presence of sugar express concentrations on a liter basis because in the development of their equation they consider a spatial arrangement of ions. The thermodynamical derivation of the relation between osmotic pressure and concentration gives $\pi = \dfrac{\dot{N_i}}{N_{H_2O} + N_i} \ RT$, an expression in which the sum of the concentrations of the ions is expressed in mol fractions, $\Sigma \ N_i$, and the mol fraction of water, N_{H_2O}. In molality terms this nearly approximates $\pi = (C) \ RT$ where (C) = mols of solute per kilo of H_2O. Many

measurements, by Rivett (1911), Morse and his associates (1915), and others, in concentrated solutions give consistent results only when calculated this way. Our own experiments are in accord with this interpretation and for these reasons we have calculated the concentrations of our experiments in mols per kilo of water.

<div align="center">EXPERIMENTAL</div>

Preparation of Solution of NaHb—Considerable experience made it evident that consistent results for Δ_{Na} could not be obtained without unusual precautions in the preparation of the solution of NaHb.

Washed horse red blood cells were electrodialyzed by the method of Stadie and Ross (1926). A mixture of 80 per cent O_2 and 20 per cent CO_2 was passed through the crystal paste which was then placed in collodion sacs and dialyzed against water saturated with O_2 and CO_2 for 24 hours.

The crystals were then washed repeatedly in ice water by stirring for 1 to 2 hours and separated by centrifugation. The conductivity of the supernatant wash water was as low as we have ever been able to obtain and the hemoglobin concentration of the

Washing No.	$K \times 10^{-5}$ mhos at 25°
10	4.6
11	3.5
12	3.3

wash water was 0.7 mM per liter.[1] To obtain a concentrated solution of isoelectric reduced hemoglobin we reduced these crystals of isoelectric oxyhemoglobin in the following way. The crystal paste was transferred to a liter saturator and reduced to Hb by 1 hour rotation in an atmosphere of CO_2 and H_2. The CO_2 was now removed by rotation for 1 to 2 hours in an atmosphere of hydrogen which was repeatedly renewed. A concentrated (11.5 mM Hb per liter) solution of reduced hemoglobin was obtained which on analysis of a 3 cc. sample showed 0.18 mM of CO_2 per liter, a negligible quantity. The freezing point of this solution was 0.006° which is practically the theoretical value with 64,000 as the molecular weight of hemoglobin. The conductivity was 5.7×10^{-5} mhos.

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[1] For convenience we retain in both papers the old convention that 1 mM of oxygen capacity = 1 mM of hemoglobin.

From this point great care was taken to avoid access of CO_2 or oxygen. The sodium hydroxide was prepared from metallic sodium. 25.00 cc. portions of the hemoglobin solution were transferred to small flasks filled with N_2 and the alkali added from a 3 cc. micro burette reading to 0.001 cc. During the addition a plentiful flow of CO_2-free N_2 into the Erlenmeyer flask was maintained. Each solution was transferred to the freezing point apparatus under a stream of nitrogen and the apparatus during the determination was kept filled with N_2. The solutions were made up as rapidly as possible and were maintained at $0°$ until used.

The water content of the stock hemoglobin was determined by drying at $110°$ and from this value and the base and water added the concentration of sodium per kilo of water calculated. The hemoglobin concentration was determined colorimetrically by the Stadie (1920) method.

The freezing points were determined by the method of Stadie and Sunderman (1931), determined in duplicate, agreement within $0.001°$ being obtained.

Preparation of NaCl-Hb Solutions—The precautions outlined above were unnecessary. Crystals of Hb from electrodialysis were washed once or twice with ice water, dissolved by addition of the requisite quantity of NaOH, and dry NaCl added by weight. The water content of each sample was determined by drying at $110°$. Determinations were made on oxy-, reduced, carbon monoxide, met-, and cyanohemoglobin solutions prepared from the crystals (Stadie and Hawes (1928)). The correction, Δ_{NaHb}, for each solution was determined before the addition of NaCl.

The freezing points of all these solutions were determined by the method of Stadie and Sunderman (1931) in duplicate, agreement to within $0.001°$ being obtained in all solutions except two where the difference was $0.002°$.

Osmotic Coefficients of NaCl in Water—In order to have values for comparison with the coefficients in hemoglobin the osmotic coefficients of NaCl in water alone were determined by our own technique. The values are given in Table I. Our values are about 1 per cent higher than those selected by Lewis and Randall (1923) from the assorted observations of Jahn, Rodebush, and Roberts and Harkins.

Results

Osmotic Coefficient of Na^+ in NaHb—The observations are of two types. In the experiment reported in Table II the Hb concentration was maintained constant and the Na concentration varied from 5.8 to 117.0 mM per kilo of H_2O. Reduced hemo-

TABLE I

Osmotic Coefficient of NaCl in Water Calculated from Freezing Points

NaCl	φNaCl
M *per kg. H_2O*	
0.05	0.957
0.10	0.943
0.20	0.933
0.30	0.922
0.40	0.918
0.50	0.913
0.60	0.910

TABLE II

Osmotic Coefficient of Na^+ in NaHb Solutions

(Hb) = 13.5 mM per kilo of H_2O

Δ_{Hb} = 0.006°

Na	$\dfrac{Na}{Hb}$	Δ_{Na} corrected	φNa
mM *per kg. H_2O*		°C.	
5.8	0.4	0.008	0.74
11.8	0.9	0.017	0.77
29.3	2.2	0.044	0.81
46.8	3.5	0.072	0.83
64.4	4.8	0.097	0.82
87.8	6.5	0.127	0.78
117.0	8.6	0.167	0.77
Mean..			0.79 ±0.027

globin was used to obtain a concentrated base-free solution to which Na could be added in varying quantities. The base-free reduced hemoglobin gave an observed Δ_{Hb} of 0.006° and this value was subtracted from the observed values of Δ for the NaHb solutions.

In the experiments given in Table III the ratio between Hb and base remained constant but by dilution the concentration of Na and Hb was reduced 3.8- and 7.0-fold respectively.

The observed freezing points were corrected for Δ_{Hb} with use of Adair's (1925) equation for osmotic pressure

$$\pi = \frac{2.55 \; C}{1 - 0.0168 \; C}$$

where $C = $ gm. of hemoglobin per 100 cc. of solution, and from which the freezing point depressions in the following tabulation were calculated.

(Hb) *mM per kg. H$_2$O*	Δ_{Hb} *°C.*
5	0.003
10	0.006
15	0.010
20	0.015

In Table II there is no consistent change of φ_{Na} despite a 20-fold change in Na concentration and Hb:Na ratio. Within the limits of the method we believe that the results show a practically constant osmotic coefficient.

In Table III the Hb:Na ratio is constant but there is a 3.8-fold and 7.0-fold change of NaHb concentration. It is possible to say that there is no change in φ_{Na}. All three experiments give for φ_{Na} approximately the same values whose mean is 0.75. Our conclusion (see Table IV) is that Na$^+$ in concentration from 5 mM to 175 mM per kilo of water as NaHb when Hb varies from 3 to 20 mM per kilo of water has a constant osmotic coefficient of 0.75. This latter value is in accord with that of Adair (1925) and of Austin, Sunderman, and Camack (1926) whose values at a few isolated concentrations of Na and Hb lay between 0.75 and 0.80. It may be pointed out here that the OH$^-$ concentrations are in all cases negligible. The most alkaline solution was one containing Na $= 175$ and Hb $= 229$. The calculated OH$^-$ of this solution would be 10^{-5} mM, a negligible quantity in its effect on Δ.

Osmotic Coefficient of NaCl in Hemoglobin Solutions—The data are given in Table V and show quite conclusively that φ_{NaCl} in hemoglobin solutions is the same as in water over a considerable range of Hb and NaCl concentration. This is in conformity with

the conclusion of Van Slyke, Wu, and McLean (1923) based on stoichiometrical analyses of whole blood.   We wish to point out

TABLE III
Osmotic Coefficient of Na^+ as $NaHb$ in Oxyhemoglobin

Hb	Na	$\Delta_{observed}$	$\Delta_{corrected}$	φ_{Na^+}
		Na: Hb = constant = 6.3		
mм per kg. H₂O	*m.-eq. per kg. H₂O*	°C.	°C.	
4.4	27.7	0.043	0.041	0.79
7.5	47.3	0.069	0.065	0.74
11.0	69.3	0.098	0.092	0.71
14.7	92.7	0.138	0.129	0.75
16.8	106.5	0.157	0.146	0.73
				0.74 ± 0.020
		Na: Hb = constant = 7.7		
3.3	25	0.036	0.035	0.75
4.6	35	0.050	0.048	0.74
8.4	64	0.086	0.082	0.69
13.1	100	0.143	0.137	0.74
15.8	121	0.175	0.165	0.73
22.9	175	0.255	0.237	0.72
				0.73 ±0.015

TABLE IV
Summary

Experiment No.	Hb	Na	$\dfrac{Na}{Hb}$	φ_{Na}
	mм per kg. H₂O	*m.-eq. per kg. H₂O*		
1	13.5*	5.8–117	0.4–8.6	0.79
2 a	4.4–16.8	28 –107	6.2*	0.74
2 b	3.3–22.9	25 –175	6.2*	0.73
Mean..				0.75

* Constant.

that since the calculations of φ_{NaCl} are made from freezing point depressions, the conclusion is without extrathermodynamic assumptions.

DISCUSSION

Activity of Na+ in NaHb—There is no quantitative theory at hand to explain the osmotic behavior of Na ions combined as NaHb. We are not concerned here with the precise mode of

TABLE V

Osmotic Coefficient of NaCl in Hemoglobin Solutions

	Hb	NaCl	Δ_{NaCl}	φ_{NaCl} in Hb	$\dfrac{\varphi \text{ NaCl in Hb}}{\varphi \text{ NaCl in H}_2\text{O}}$
	mM per kg. H₂O	*mM per kg. H₂O*	*°C.*		
HbO₂	8.0	85	0.298	0.95	1.00
	8.0	203	0.703	0.93	1.00
	16.8	276	0.814	0.80	(0.86)
	9.0	296	1.022	0.93	1.01
	8.0	466	1.599	0.92	1.01
	26.3	485	1.738	0.96	1.06
					1.02
RHb	7.5	312	1.057	0.91	0.99
	9.6	341	1.177	0.93	1.01
					1.00
HbCO	21.4	274	0.975	0.96	1.03
	25.8	614	2.124	0.94	1.03
					1.03
HbCn	5.7	226	0.801	0.95	1.03
	13.3	298	1.001	0.90	0.98
					1.01
MtHb	9.8	270	0.952	0.95	1.03
	14.8	356	0.211	0.92	1.00
	14.3	370	1.246	0.91	0.99
					1.00
Mean of all...					1.012 ±0.005

chemical combination but it is possible to say that it is of such a nature that the Na+ can diffuse sufficiently far into the solvent so as to exert a considerable osmotic pressure. In other words NaHb is ionized and for simplicity we may regard it as completely ion-

ized. We can further say that the attracting and repulsive forces and the interaction of adjacent portions of the hemoglobin molecule upon Na^+ ions influence them in such a way that the osmotic coefficient is diminished to 0.75. This, of course, is in conformity with our general expectations in compounds of this character.

The explanation of the fact that marked changes in the Na : Hb ratio and changes of concentration of NaHb do not influence φ_{Na^+} is readily found in a consideration of the huge discrepancy in the size of the combining masses. The compound NaHb can be pictured as one in which a few small sodium ions are distributed in a more or less uniform manner over a huge hemoglobin molecule. In such a case the interionic distances between the sodium ions would be so great that each ion may be considered thermodynamically isolated with respect to other sodium ions. Ionic interaction between sodium ions would be nil and therefore changes in the ratio Na^+ : Hb or the concentration of NaHb would be without influence on the osmotic coefficient activities so that φ_{Na^+} would be independent of these changes. We believe that our experiments show that NaHb cannot be regarded as a high valence salt of the type Na_nHb in which ionic interaction would be marked and would produce profound effects on the osmotic properties, with changes of concentration. In this respect our conclusions are in conflict with the views of Adair (1928) who has measured the activity coefficient f_{Cl} of the chlorine ion in solutions of edestin chloride by means of membrane potential measurements and found a decrease of f_{Cl} from 0.86 to 0.59 with roughly a 4-fold change in concentration of Cl and a 13-fold change of edestin concentration. On the basis of this experiment Adair regards edestin as a multivalent protein ion of valence 24; further he calculated f_{Cl} by the Debye-Hückel equation in the form

$$- \log f_{Cl} = \frac{0.34 \, J^{\frac{1}{2}}}{1 + 0.69 \, J^{\frac{1}{2}}}$$

where J is the ionic strength and is calculated by the equation

$$J = [H] + [Cl] + n_p^2 \, C_p$$

where $n_p = 24 =$ valence of edestin and $C_p =$ molecular concentration of protein using 17,000 as the molecular weight. He obtains plausible agreement with observed values.

Now, in a general way edestin chloride is analogous to NaHb both being ionized into a simple ion and a complex protein ion and it would be expected on the basis of this analogy, that the thermodynamic behavior would be the same; yet the electromotive force determinations of f_{Cl} in edestin chloride show an effect of protein which is absent in the osmotic determination of φ_{Na}.

Several serious difficulties must be overcome before the application of the quantivalent notion of the protein ion in the calculation of J, the ionic strength, can be accepted on the basis of Adair's experiments with edestin chloride. One has already been discussed in the preceding paragraph. Another is the fact that Adair's ratios of $\dfrac{n_p^2\,C_p}{J}$ are high (0.78 to 0.95) and roughly constant so that $J = $ constant C_p. That is to say, the molecular protein concentration is the major factor in his equation for calculating J and therefore f_{Cl} and his calculated values would have an entirely different magnitude if a molecular weight other than 17,000, Adair's value, was used for edestin. Since Cohn (1925) gives 29,000 as the minimal and 58,000 as the probable molecular weight of edestin, a simple recalculation from Adair's data using 58,000 causes the agreement between calculated and observed values to disappear. Again the number of charges per hypothetical ion of edestin (using 17,000) as calculated from Adair's ratios of $[Cl]:C_p$ vary from 31 to 100. Nevertheless, Adair regards the number as constant at 24. Logically a variable n_p value should be used in the calculation of J but in such a case the agreement of f_{Cl} with observed values again disappears.

Besides this internal evidence in Adair's experiments which must be considered before his conclusions are accepted, there is some direct experimental evidence particularly designed to throw light upon this question of quantivalence. Stadie (1928) showed that a considerable change in the number of charges upon the hemoglobin molecule was without effect upon the activity coefficient of the bicarbonate ion and concluded that hemoglobin could not be regarded as a quantivalent ion in its effect upon the ionic strength. Again Simms (1928) has subjected the question to a careful analysis and has shown that even simple multicharged particles no longer behave as quantivalent ions in respect to ionic strength when the distance between charges exceeds a certain

distance. These considerations lead us to doubt whether Adair has established the notion that charged protein ions behave as quantivalent ions in their effect on the ionic strength and to consider the extension of the Debye-Hückel theory to protein solutions by the application of this hypothesis as hazardous.

Activity of NaCl in Hemoglobin Solutions—The experiments on the osmotic coefficient of NaCl in hemoglobin solutions over a very wide range of concentration lead unequivocally to the conclusion that hemoglobin is without effect on the osmotic properties of NaCl. Since this conclusion is arrived at completely without extrathermodynamic assumptions it must receive considerable weight. Considerations of the following character immediately lead us to a dilemma from which we can suggest no certain escape.

There is a considerable amount of data derived entirely either from membrane or single electrode potentials which indicate that proteins influence the activities of ions. Thus Pauli and Schön (1924) and Northrop and Kunitz (1926) attributed the discrepancy between electrometric and stoichiometrical concentrations to the formation of an unionized protein chloride complex. Later Pauli and Wit (1926) adapted the complete ionization hypothesis and concluded that the activity coefficient of chloride ion is changed by protein. Van Slyke, Hastings, Murray, and Sendroy (1925) calculated a difference in activity coefficients of the chloride ion and the bicarbonate ion in red blood cells and plasma, the ratios being 0.77 and 62 respectively. Stadie and Hawes (1928) showed a systematic effect of hemoglobin upon the activity coefficient of the bicarbonate ion. Adair (1928) also, in membrane equilibrium experiments showed a diminution of chloride ion activity in $NaCl^-$ hemoglobin solutions. It is true that in every case the conclusions are limited by certain assumptions, the most serious and usually the only ·extrathermodynamic one being that which eliminates the liquid junction potential.

Harned (1924), Taylor (1927), Güntelberg (1928), and others have repeatedly pointed out the uncertain validity of this assumption and though biochemists have been well aware of this, nevertheless, they have tended to ignore it in drawing their conclusions. Since the magnitudes of liquid junctions are completely unknown, especially in protein-containing solutions conclusions drawn from electrometric measurements involving them must be made with

some reserve. Freezing point and electrometric measurements of ion activities are both based on thermodynamic considerations and hence should give precisely the same results, as has been amply demonstrated in simple aqueous solution (Lewis and Randall, 1923).

These considerations would make it appear that our present knowledge of the activity of ions in the presence of proteins may be summed up in a general way in two statements: (1) The mean ion activity coefficient of a salt (*e.g.* NaCl) dissolved in a protein solution is 1.0 with respect to its value in pure water at the same molal concentration; whereas (2) the single ion activity coefficient of one of the ions (*e.g.* Cl⁻) is systematically changed from its value at the corresponding concentration in water by increasing concentrations of protein.

These two conclusions can be shown to be contradictory by a consideration of what is measured in the determination of the two types of activity coefficients.

In the case of the mean ion activity of the salt it is clear that it is the free energy change from the given state to the standard state (activity = 1.0) which is measured. If for convenience we take as the standard state a solution of the salt in pure water at any given molal concentration, the equation for this free energy change is

$$F_{\pm} - F'_{\pm} = RT \ln \frac{f_{\pm}^2 \, C}{f'^2_{\pm} \, C}$$

where $F_{\pm}$, $F'_{\pm}$ are the free energies of the salt in the given state (with protein) and standard state, $f_{\pm}$ and $f'_{\pm}$ the respective mean ion activity coefficients, and C is the molal concentration. If the first generalization of the preceding paragraph is true it is evident that since $f_{\pm} = f'_{\pm}$

$$F_{\pm} - F'_{\pm} = 0$$

when passing from a protein solution to pure water at the same molal concentration.

The fact that the mean ion activity coefficient is calculated from the freezing point depression does not alter the validity of

this argument since such a method measures the free energy of the water which is related to that of the salt by the relation

$$F_{NaCl} = - \frac{N_{H_2O}}{N_{NaCl}} F_{H_2O}$$

where N_{H_2O} and N_{NaCl} are the respective molar fractions. Nor does the fact that we have a third constituent (protein) in the solution hamper our ability to determine F_{NaCl} if in the equation

$$N_{H_2O}F_{H_2O} + N_{NaCl}F_{NaCl} + N_{Hb}F_{Hb} = 0$$

for the free energy of the system we can be reasonably sure that changes in $N_{Hb}F_{Hb}$ are zero or negligibly small.

In the case of single ion activity coefficients the situation is not quite so clear. The presumption is that what is measured is the change of free energy of the ion in passing from the given state to the standard state. With a notation analogous to the previous case, this free energy change of this operation is expressed by the equation

$$F_- - F'_- = RT \ln \frac{f_-}{f'_-}$$

F_- and f_- are the free energy and activity coefficient of the ion in the protein solution while F'_- and f'_- are the corresponding quantities in pure water at the same molal concentrations. It is a moot point whether such an operation has any meaning since it is questionable whether it is possible to transfer an ion from a given state to a standard state so that the measurement may be one of a process which has no reality. Indeed, Taylor (1927) has published a mathematical proof that such a process is expressible in terms of molecular free energies only and hence cannot be made to yield any information concerning individual ion activities. This difficulty need be no deterrant to handling the quantity, at least as a mathematical device and indeed much valuable information particularly in biological fields has been obtained by its free use. The significance, then, of the second statement above would be that since f_- is not equal to f'_- $F_- - F'_-$ is not zero but finite. Since, how-

ever, the partial free energy of the salt is equal to the sum of the free energies of the ion in both given and standard state we have

$$F_{\pm} = F_{+} + F_{-}$$

$$F'_{\pm} = F'_{+} + F'_{-}$$

from which

$$F_{\pm} - F'_{\pm} = (F_{+} - F'_{+}) + (F_{-} - F'_{-})$$

Since

$$F_{\pm} - F'_{\pm} \text{ is zero}$$

$$F_{+} - F'_{+} = - (F_{-} - F'_{-})$$

which can only be true if both are zero or the increase of the free energy of one ion by protein is always equal to the decrease of the free energy of the other. This would presuppose the existence of some new and unique balancing thermodynamic mechanism which is extremely unlikely.

In brief we believe that the osmotic determinations of φ_{NaCl} in protein solutions and the electrometric and membrane potential determinations of the activity coefficient of Cl^{-} by Pauli, Adair, and others are sharply contradictory.

We have no solution of the problem nor do we state without reserve that the difficulty is a real one. It is quite possible that with a greater knowledge of the effect of proteins upon the thermodynamic properties of single electrodes and on the liquid junction potential, the situation will be clarified. We can only emphasize again the statement that osmotic and electrometric determinations of activity coefficients must agree.

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DETERMINATION OF THE pH OF SERUM AT 38° WITH THE GLASS ELECTRODE AND AN IMPROVED ELECTRON TUBE POTENTIOMETER*

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INTRODUCTION

The preparation of a glass (MacInnes and Dole, 1930) having nearly perfect electrical properties for use as glass electrodes to determine pH over a wide range and the use of a stable and sensitive electron tube potentiometer (Stadie, 1929) for the determination of E.M.F. makes the glass electrode method for the determination of pH easily available for those familiar with ordinary potentiometric technique. The advantages of the glass electrode in economy of material and ease of manipulation have been fully discussed in the literature. It is possible to say now that the glass electrode gives a degree of accuracy in the determination of pH of serum not achievable by the hydrogen electrode.

In this paper we report adaptation of these developments particularly to serum together with a modification of Stadie's electron tube potentiometer giving increased accuracy and stability as well as ease of manipulation.

Construction of a Glass Electrode Suitable for Determination of Serum pH at 38°

For serum, blood, and other CO₂-containing solutions, it is of course necessary to construct the electrode to avoid loss of CO_2 during the filling and reading. We have found the electrode pictured in Fig. 1 to be highly satisfactory in this respect. A heavy walled pipette of 1 mm. inside bore has a small bulb (0.2 cc.)

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* Reported to the Physiological Society of Philadelphia, May 19, 1930 (Stadie and O'Brien, 1930).

fitted with a side tubulation which is fused into a jacket as shown. The jacket which measures about 50 × 110 mm. serves as a constant temperature bath by which the electrode is maintained
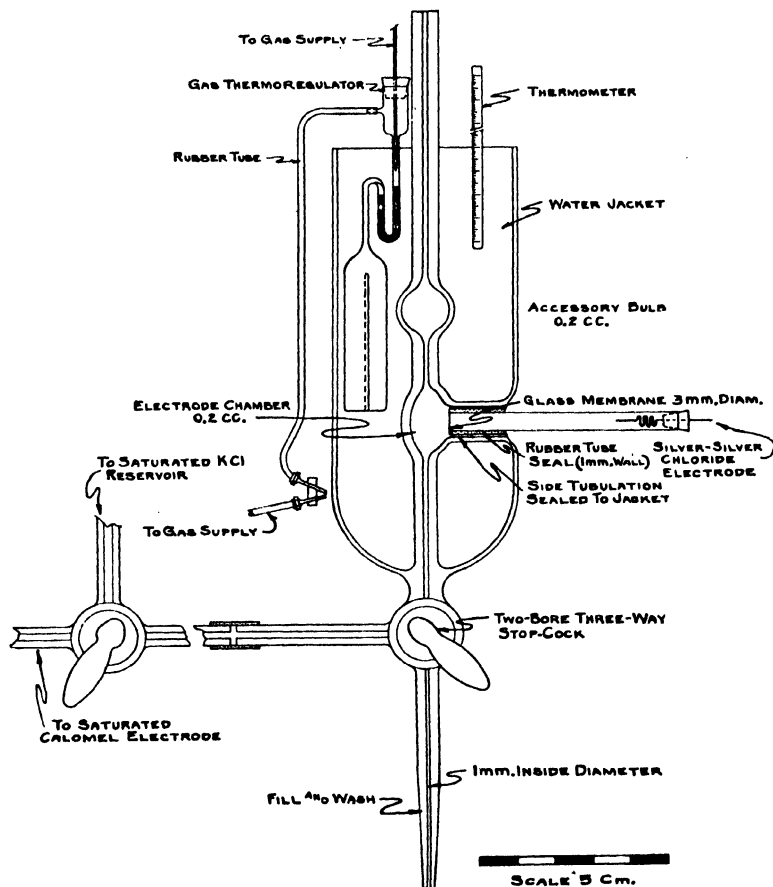
FIG. 1. Assembly of electrode thermoregulator and connections to calomel cell.

at 38°. Above the first bulb is an accessory bulb of 0.2 cc. capacity without tubulation, whose function will be described below. Into the side tubulation is fitted by means of a bit of rubber tubing

(3 mm. inside diameter, 1.0 mm. wall) a glass electrode which is made in the manner described by MacInnes and Dole (1930). At the lower end of the jacket is fused a 3-way 2-bore stop-cock (Fig. 1a) which in three positions accomplishes the following: (1) allows of filling or washing electrode chambers, (2) establishes a liquid junction of the serum in the electrode and the saturated KCl in the side arm leading to the calomel electrode, (3) allows of flushing out by saturated KCl from a reservoir the old mixture of serum and KCl preparatory to making a fresh KCl junction. A 3-way stop-cock to the left permits connection to a small reservoir containing saturated KCl or to the saturated calomel electrode. The latter is completely filled with KCl and tightly stoppered. In conse-
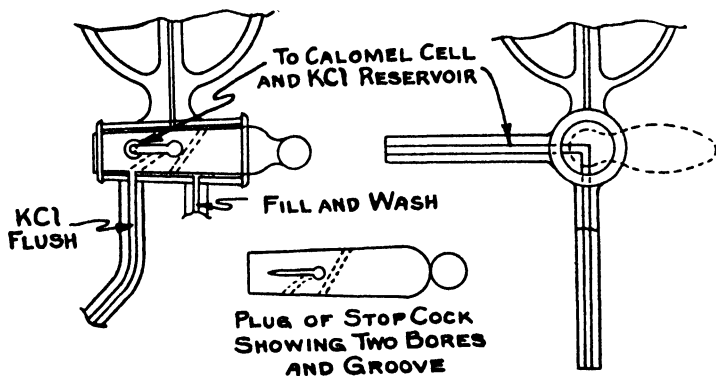
FIG. 1a. Detailed view of 3-bore 4-way stop-cock showing the various interconnections.

quence the salt bridge is closed and there can be no flow of KCl when the junction is established. This stop-cock arrangement is a modification of the stop-cock designed by Simms (1923) for his water jacket hydrogen electrode which we have used with great success in this laboratory. This assembly made a sturdy piece of apparatus which may be suitably clamped in position, washed, and filled with ease. We have used two sizes of electrodes. One, with a membrane 5 mm. in diameter, contains 0.5 cc. exclusive of the accessory bulb, and a second with a membrane 3 mm. in diameter, having a volume of 0.2 cc. exclusive of the accessory bulb. For pH determination on serum, then, the required amounts are about 1

to 5 cc. and 0.5 cc. respectively which amounts allow for ample
wastage. For routine work the smaller electrode is to be pre-
ferred for economy of material and accuracy.

Temperature Control

The sensitivity of the grid to induction from the make and break
of electrical current make it better to avoid electrically controlled
heating elements and to use a simple gas heater which maintains
the bath at $38° \pm 0.05°$. The bath consists of the jacket described
above which is filled with water. It is maintained at $38°$ by a small
gas thermoregulator which contains toluene and mercury after
the usual fashion and which controls the flow of gas to a small
flame at the blunt end of a 22 gauge Luer needle. A small pilot
flame from a second needle is used to ignite the intermittent flow
of gas from the thermoregulator. The two Luer needles are
thrust through a cork with their tips in juxtaposition and sup-
ported so that the small flames which are protected from drafts
by a small cylinder of metal just impinge upon the outside of the
water jacket. The thermoregulator together with a thermometer
and an air inlet tube (not shown) introducing compressed air for
stirring the water are supported in the jacket by a large stopper.
The arrangement is shown in Fig. 1.

Connections to Electrometer

Into the projecting end of the side tube is fitted an $Ag \cdot AgCl$
electrode held in place by a small cork as shown in Fig. 1. The
side arm is filled with 0.1 N HCl and by a small slit in the cork,
this connection is made non-air-tight, to prevent rupture of the
glass membrane by expansion of the HCl with changing tem-
perature. The second connection to the electrometer is made
through a saturated KCl junction established in the side arm of
the lower stop-cock to a saturated calomel half-cell in the manner
described above. Again Fig. 1 shows this assemblage.

Preparation of $Ag \cdot AgCl$ Reference Electrode

Any standard method, of course, is satisfactory, but since the
electrode serves merely as a reference source of E.M.F., constancy
alone is desired so that no great care is needed in its manufacture.
The following simple method suffices. A bit of No. 20 to 22 silver

wire wound into a helix is stuck through a small cork and is plated with a coating of colloidal silver by electroplating from a silver cyanide solution. It is washed and then electrolyzed in a solution of 0.1 N HCl. A brownish or purplish coating of AgCl results which gives a quite stable E.M.F. in 0.1 N HCl of approximately 0.042 volts when combined with a saturated calomel half-cell.

Electrometer Circuit

Since the publication of the original description of the balanced Wheatstone bridge circuit (Stadie, 1929), two papers, that by
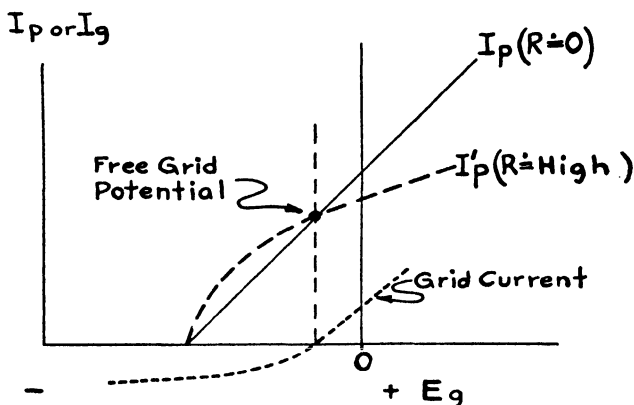
FIG. 2. Schematic representation of the plate current and grid current-grid volts characteristics of the screen grid tube to show the advantages of working at the free grid potential.

Nottingham (1929) and by Rasmussen (1929), have elucidated the nature of the grid current characteristic of electron tubes, and have made it clear that the advantages of selecting the so called "free grid potential" as the operating point of an electron tube are sufficient to warrant the slight complication of the circuit necessary to accomplish this. Accordingly we have modified the circuit so that instead of working at a fixed negative bias of −1.5 volt on the grid, it is possible by the introduction of simple potential dividers in the grid circuits to operate the tubes at free grid potential. The above papers should be consulted for details of the theory involved. It is sufficient here to state the following brief considerations which bring out the advantage of operating the tube at the free grid potential.

Fig. 2 employs the usual terminology (see Stadie (1929) and Clarke (1928)) for plotting electron tube characteristics and gives schematically the plate current and grid current of SX 232 plotted against grid bias.

E = grid potential.
I_p = plate current-grid bias characteristic with low grid resistance.
I_p' = same with high grid resistance (*e.g.* glass electrode).
I_g = grid current characteristic. The portion below the 0 line is the positive ion current.

Free Grid or Floating Potential—The grid of an electron tube which is completely insulated or free will assume a potential whose value depends upon the geometry of the tube, the amount of residual gas, and the operating conditions. In general any factor which decreases the ionization of residual gas such as hardness and diminished current to the plate will increase the negative value of the free grid potential. From the point of view of the operating characteristics the free grid potential may be defined in several ways. (*a*) It is the intersection of the two (low and high grid resistance) plate current characteristics. (*b*) Its ordinate is the plate current when the grid is free, *i.e.* when R_g (the resistance in the grid) is infinite. (*c*) The corresponding abscissa is the free grid potential, and is usually negative with respect to $A-$. If a potential divider is set at the value of the free grid potential and the grid is connected to $A-$ through the divider, the tube is said to be operated at the free grid potential. (*d*) It is the point where the negative electron grid current changes over into the positive ion (gas) grid current, *i.e.* the point where there is no grid current. It is evident from the diagram that when operated at the free grid potential there will be no change of plate current when (*a*) the grid is free or (*b*) connected to $A-$ through high or low grid resistance.

The advantages of operating at the free grid potential are now apparent. If a cell of high resistance such as a glass electrode is placed in series with a student or other potentiometer so that the E.M.F. of the two are exactly opposed, the two together will be resistance only and there will be no change of plate current when the grid is (*a*) free or connected to $A-$, (*b*) directly through the potential divider, or (*c*) indirectly through the glass cell. This

will be the null or zero position and a sensitive galvanometer in the plate circuit will show no deflection.

Since at balance the grid current is precisely zero (not merely vanishingly small) no current will be drawn from the cell and hence there will be no $I_g R$ drop in the grid circuit so that the reading of the potentiometer used to balance out the glass cell will be the true E.M.F. of the cell. Small diurnal changes in the plate current characteristic are immediately compensated by readjustment of the grid bias potential divider.

As a matter of fact zero grid current would only be attained if the potential divider in the grid circuit used to maintain the tube at free grid potential and the potentiometer were infinitely divisible. Now it can be shown that the grid current is

$$I_g = \frac{V - E_{fg}}{R_e + R_i}$$

where $V - E_{fg}$ is the voltage difference from the free grid potential, R_e the external grid resistance (50 to 300 $\times$ 10^6 ohms for average glass electrode), and R_i is the internal grid to filament resistance (10^{10} ohms for a good tube of the SX 232 type). Hence for 0.1 millivolt E.M.F. which is the limit of the student potentiometer and the grid potential divider we have at balance

$$\frac{10^{-4}}{0.3 \times 10^9 + 10^{10}} = 10^{-14} \text{ amperes}$$

approximately. This value is to be compared with 1000 $\times$ 10^{-14} for high sensitivity galvanometers which are as a rule unstable and difficult instruments to handle.

The diagram of the circuit appears in Fig. 3. It is essentially the same as the first circuit (Stadie, 1929) except for the insertion of a potential divider in the grid circuit of one tube in place of a fixed bias of 1.5 V and the elimination of any grid connection on the second tube. A list of components is appended. We have found that a shield made of brass or aluminum enclosing the assembled units as indicated by the dotted lines on the diagram is essential and assures complete freedom from stray electrical effects. The glass electrode, calomel half-cell, standard cell, etc. need not be shielded.
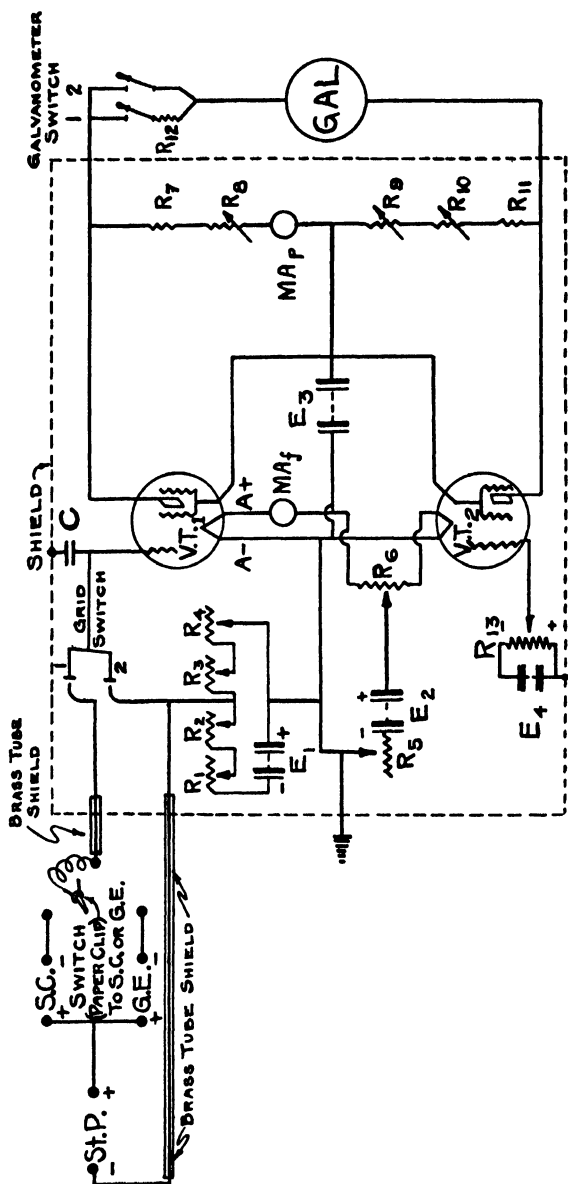
FIG. 3. Details of the modified balanced Wheatstone bridge circuit designed to operate at free grid potential

Components

St.P.	Leeds and Northrup student potentiometer with its battery and resistances
S.C.	Marion Eppley standard Weston cell
G.E.	Glass electrode with connecting calomel and Ag·AgCl electrodes. Small paper clip on extended grid lead which can be clamped to leads from standard cell to glass electrodes at will
Grid switch *1, 2*	3-Way spring wire tapping switch, Fig. 5
R_1, R_4	200 ohm Yaxley junior potentiometer used as a rheostat
R_2	400 ohm Yaxley potentiometer
R_3	40 ohm Yaxley rheostat
R_5	40 " " "
R_6	6 ohm Yaxley junior potentiometer
R_7, R_{11}	Durham 12,500 ohm Powerohm
R_8, R_{10}, R_{13}	2000 ohm Yaxley junior potentiometer used as a rheostat
R_9	400 ohm Yaxley potentiometer
R_{12}	50,000 ohm Durham resistor
E_1, E_4	1.5 volt dry cell
E_2	6 volt storage battery
E_3	45 volt Burgess heavy duty B battery
MA_f	Weston No. 506 *S* 200 milliammeter
MA_P	" " 506 " 1.5 "
V.T. $_1$, *V.T.* $_2$	Sylvania screen grid tubes
Gal.	Leeds and Northrup galvanometer No. 2420 C.
Galvanometer switches	Yaxley midget battery switch
C	0.003 micro farad mica condenser

The various units of the apparatus are assembled upon a bakelite chassis in the usual way. The front panel of the chassis is covered by an aluminum sheet and is pierced by the controls of units R_2, R_4, R_8, and R_9. Upon it are also mounted a filament, switch, and the two galvanometer switches, and in addition the filament milliammeter (MA_f) and plate milliammeter (MA_P). The grid switch tapping levers are mounted toward the left (Fig. 5). All other resistance units (R_1, R_4, R_5, R_6, R_7, R_{10}, R_{11}, R_{12}) which are infrequently used are mounted upon a raised bakelite saddle at the rear of the subpanel. The tubes are mounted at the extreme ends of the subpanel. Most of the wiring is beneath the subpanel, so that the entire arrangement is neat and compact. The chassis is enclosed in an aluminum box pierced at one end by brass tubes covering glass tubes through which the grid leads are threaded.

It is essential that the tube and operating conditions be selected
with care and a knowledge of the behavior of the tubes and circuit
in order to secure stability and sensitivity.

The guiding principles outlined below are in part discussed in
standard text-books (Morecroft) and in part are the results of our
experience and are given in brief outline.

The mutual conductance $G = \dfrac{\Delta I_p}{\Delta E_g}$ when the grid resistance is
zero is related to the similar quantity G' when the grid resistance
is high by the equation

$$\frac{G'}{G} = \frac{R_i}{R_i + R_e}$$

where R_e is the external grid resistance and R_i is the input resis-
tance or the internal (grid to filament) resistance. The important
quantity R_i determines the worth of the tubes and under the same
operating conditions may vary several thousand-fold for tubes of
the same type. If insulation leakage be disregarded the input
resistance depends inversely upon ionization of residual gas in
the tube. If, therefore, by the use of special precautions in evacu-
ation and a highly active getter, the residual gas is reduced beyond
ordinary limits, the tubes are of such hardness and have such high
R_i values that they can be used satisfactorily with such extremely
high resistances as 5000 to 100,000 megohms in the external grid
resistances. In consequence, the comparatively small resistances
of 150 to 300 megohms of a well prepared glass electrode of 3 mm.
diameter function practically as if no resistance was in the grid
circuit.

To determine R_i the following steps are necessary. (1) Deter-
mine S, the current sensitivity of the galvanometer by applying
a small E.M.F. from a student potentiometer through a known
resistance. Then

$$S = \frac{E}{R_d}$$

where d is the galvanometer deflection in mm. (2) Absolute
alcohol-xylol resistors (Campbell, 1912) of a 1 to 10 mixture in

closed tubes with fused-in Dumet or other metal leads are cali-
brated by applying a large E.M.F. to the resistance in series with the
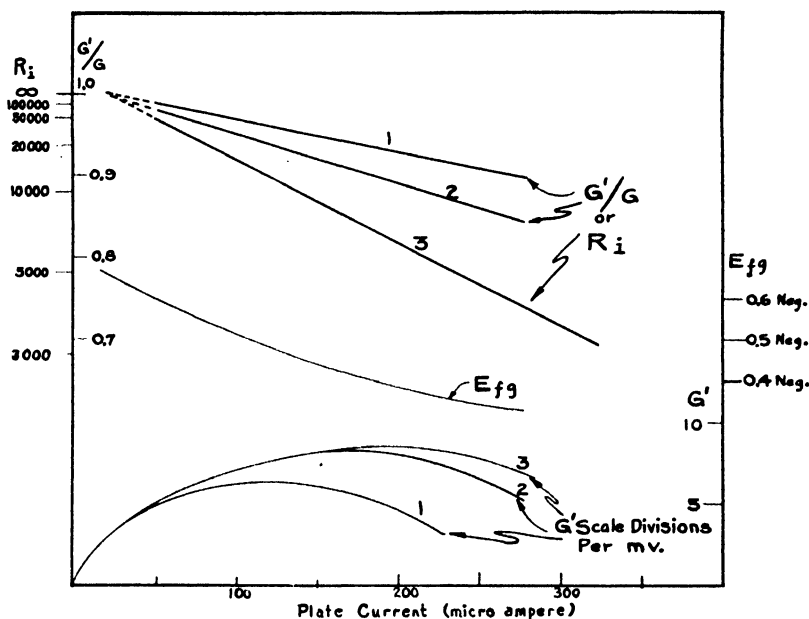
FIG. 4. Characteristics of the Sylvania SX 232 tube. The external grid
resistance was a 1400 megohm alcohol-xylol resistor. The filament current
was constant at 46 milliamperes. E_p for Curve 1 = 129 volts, Curve 2=
85 volts, Curve 3 = 45 volts. The plate current is plotted as abscissæ
and was varied by varying the screen grid potential. The upper curves
show the variation of $\dfrac{G'}{G}$ and R_i as the plate current is decreased. At
$\dfrac{G'}{G}$ = 1 the 1400 megohm resistor had no effect on G; i.e., the external grid
resistance was infinitely great compared to it. The middle curve shows
the increase of negativity of the free grid potential and the lower curves
show changes in the effective mutual conductance G' measured in mm. of
galvanometer deflection per millivolt change of E_g.

galvanometers. Then $R_e = \dfrac{E}{S_d}$. Several resistors from 1000 to
10,000 megohms are needed. (3) The tube to be tested is placed
in the first socket and the plate current balanced. At free grid

potential and with no added grid resistance the G is determined in
mm. of deflection per millivolt using the student potentiometer to
impose the ΔE_g. G' is similarly determined using the known alco-
hol-xylol resistor of 2000 to 10,000 megohms. R_i is calculated by
the equation

$$R_i = \frac{G'}{G - G'} R_e$$

An important property of the screen grid tube of which full use
is made at this point is that if filament current and plate potential
are constant a decrease of the screen grid potential gives an in-
crease of the internal grid resistance without a corresponding
decrease of effective mutual conductance.   In this way it is pos-
sible to increase the internal resistance a thousand times at a cost
of only one-half of the effective mutual conductance.   The tubes
to be used are systematically tested by maintaining filament cur-
rent and plate potential constant while screen grid potential is
varied.   A plot of $\dfrac{G'}{G}$ is made against the plate current and the
determinations repeated for another set of conditions.   In this
way it is possible to determine at once whether the tube is a good
one and just the proper conditions to select for operation.   The
accompanying chart (Fig. 4) shows for one tube the limits of the
factors R_i, G', and I_p.

Several tubes equalling the above in hardness and in the very
high input resistance were obtained from the Sylvania Products
Company of Emporium, Pennsylvania through the courtesy of
Roger M. Wise, Chief Engineer.

Operation of Electron Tube Potentiometer

There are three subsidiary procedures which are frequently
made in the various steps of the operation of the electron tube
potentiometer during the determination of pH of serum or blood.

1. Balancing the Plate Currents to Null Position of Galvanometer—
Both grids being free, the resistances R_8 to R_{11} in the plate circuit
are varied until a position is found where no current flows through
the galvanometer (Gal).   During this operation, galvanometer
switch *1* with its 50,000 ohms resistance is used to put the galva-
nometer in the circuit until approximate balance is found and then

switch *2* (without resistance) is used for final fine adjustment. The galvanometer image is now at zero indicating no current through the galvanometer. From time to time during the day adjustments are made to return the galvanometer image to the zero position. The amount of drifting of the galvanometer image, however, is very small ($<$ 1 mm. per hour) so that these adjustments are infrequent. Moreover, there is no necessity for the image to be exactly at zero but any convenient position on the scale may be taken as the null point.

2. Grounding the Grid to A — —(*a*) *Directly* through the potential divider (E_1, R_1 to R_4). By key *2* of the grid switch the grid of the upper tube is connected to A — through a fraction of the E.M.F. of E_1 determined by the setting of the resistances R_2 to R_4. The grid potential selected is of course the free grid potential (*vide infra*). (*b*) *Indirectly* through the high resistance of glass electrode, student potentiometer, and the grid potential divider (E_1, R_1 to R_4). Key *1* of the grid switch makes this connection.

The various steps in the operation of the electron tube potentiometer may now be described in terms of these subsidiary ones.

(*a*) *Adjusting the Filament Potential Divider*—This device is adapted from the scheme of Williams (1928) and minimizes the disturbing effects of changes in the filament battery E_2. It consists of a 6 ohm Yaxley potentiometer R_6 which serves as a potential divider. The adjustment which need not be exact and requires only occasional (weekly) rechecking for the same tubes at the same filament current is made as follows: Fix the slider of R_6 at the mid-point, adjust the filament current and the potential on screen grids and plates to the operating valves, and balance the galvanometer for no deflection by means of the plate resistances R_8 to R_{11}. Change the filament current slightly and note the deflection of the galvanometer on closing galvanometer switch *1*. Change the filament current back to its original value, adjust the slide of R_6 to a new position, and repeat the above operation. Depending on the magnitude and direction of the deflection further adjustments of the slider of R_6 are made until a position is found where there is only a small ($\pm$10 mm.) deflection on changing the filament current by 0.01 ampere. Both grids are free during the operation. A small graduated disc of paper under the knob of R_6 aids in the location of the point of least deflection.

(b) *Adjusting the Grid Potential Divider to the Floating Potential—* With the tubes at the proper operating conditions, adjust the plate current to the null position of the galvanometer (Procedure 1 above). Both grids are free during this operation. When the final fine adjustment of the plate resistances is made, close galvanometer switch *1* and by means of key *1* of the grid switch ground the grid of the upper tube $(V.T._1)$ directly with $A-$ through the grid bias potential divider and adjust the resistances R_1 to R_4 until there is no deflection on passing from free to closed grid. Close galvanometer switch *2* and make the final fine adjustments. The grid is now at free grid potential. The second tube $(V.T._2)$ is now brought to free grid potential in a similar way. No switches are needed but the tap of R_{13} is merely attached to the grid by a small battery clip and R_{13} adjusted until the galvanometer image has been restored to its former position. This adjustment need not be exact and requires infrequent checking.

(c) *Adjusting the Student Potentiometer—*By means of the small paper clip connect the grid to the standard cell and potentiometer with its resistance into the grid circuit. The potentiometer is set at the value of the standard cell. Ground the grid (a) directly or (b) indirectly (through the standard cell and potentiometer) until by adjustment of the student potentiometer resistances, a point is found where there is no deflection of the galvanometer image for the alternations. If the source of E.M.F. for the student potentiometer is two dry cells and these are left in circuit day and night, the need for calibrating the student potentiometer is reduced to once, or at most, twice a day. Our experience is that once the dry cells have settled down, the drift over the course of a day is about 0.1 millivolt.

(d) *Determination of E.M.F. of Glass Cell—*The student potentiometer being now properly calibrated, the standard cell is taken out of the circuit and replaced by the glass electrode by means of the clip. By the grid switch connect grid (a) directly to grid bias potential divider or (b) through glass electrode and student potentiometer, and by adjustment of the student potentiometer the point is found where no deflection of the galvanometer occurs for the alternatives (a) and (b). From time to time adjustments of the resistances R_2 and R_3 of the grid bias potential divider are made to keep the tube at free grid potential.

TOP VIEW

SIDE VIEW

SCALE 5 cm.
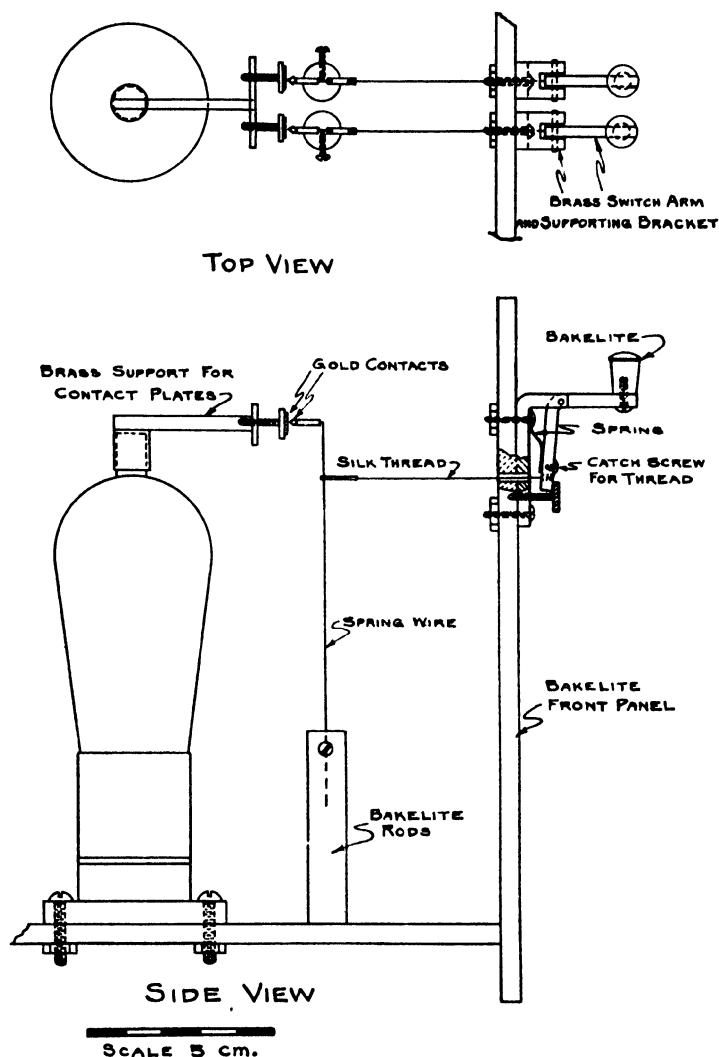
FIG. 5. 3-Way grid switch designed to ground grid of control tube to A — through the grid potential divider (a) directly or (b) indirectly through the glass electrode.

3-Way Grid Switch

The switch shown in Fig. 5 is the result of an evolutionary development from a half dozen forms and for simplicity and per-

fection of function is eminently satisfactory. A bakelite rod supports a length of spring wire bent and tipped with a gold contact point as shown. This touches a small circular plate of gold soldered upon an adjustable screw and supported upon the projecting control grid as detailed. The spring wire is held back about $\frac{1}{64}$ inch by a silk thread attached to a tapping key by a small screw. The arrangement is easily adjustable and when the tapping key is depressed a delicate but sure contact is made by the release of the spring wire which gives no false movement of the galvanometer image when at the null point.

Shielding Insulation and Capacity of Grid

The insulation of all leads to the grid, particularly those up to the glass electrode itself must be made of a high order by the use of rods or tubes of Pyrex glass, and where needed sulfur or paraffin blocks. Long leads from the grid are readily insulated and shielded by running them through Pyrex tubing over which is slipped brass tubing. The best arrangement is to place the glass electrode about 6 inches from the electron tube potentiometer and to keep the grid lead on the high resistance side as short as possible. There must be at least 4 inches of glass tubing projecting out from each end of the brass tube shield to insure complete insulation of the grid lead from ground. The loose end of the grid lead to which is attached a small clip to alternate the glass electrode and the standard cell in series with the grid lead need not be shielded. Insulation on the low side (after the glass electrode) need not be of a high order nor need the potentiometer be shielded. It is important to have the galvanometer critically damped by inserting the proper resistances in series or in shunt with the galvanometer. The natural capacity to ground of the grid is so small that any external capacity coming within its neighborhood changes it causing the grid to vary its charge and potential with resultant adventitious movements of the galvanometer image. This is the cause of the annoying hand capacity effects and the false movements of the galvanometer image when the external grid leads are thrown into the grid circuit by the grid switch. These difficulties disappear if the grid capacity is increased by connecting a small mica condenser of about 0.002 to 0.004 micro farad capacity from grid to ground as in C of Fig. 3.

General Characteristics of Electron Tube Potentiometer

The apparatus described above is a decided improvement over the original design. The action is very steady and the drifting of the zero position of the galvanometer image is practically eliminated being less than 1 mm. per hour. The value of the floating potential also changes very slightly during the course of the day.[1] Once the tubes have reached a steady state (15 to 20 minutes) the adjustments of plate resistances and grid potential divider are infrequent and slight.

Most important is the fact that the true E.M.F. of the cell in the grid circuit is measured without drawing current from it. The sensitivity with 45 volts on screen grid and plates and a total filament current of 0.250 amperes is about 10 to 15 mm. of scale deflection per 1 millivolt. This can be increased to as high as 15 to 25 mm. of deflection per millivolt by increasing the plate voltage to 90 volts at slight cost of stability. The lower sensitivity is however ample.

We have had no difficulty in reading the E.M.F. of glass electrodes to 0.1 millivolt using a student potentiometer.

Calculation of pH and Determination of e, the Glass Cell Constant

For the cell

$$\text{Hg, HgCl saturated KCl} \left| \begin{array}{c} \text{Sol.} \\ a_{\text{H}} = 10^{-\text{pH}} \end{array} \right| \text{Glass} \left| 0.1 \text{ N HCl AgCl} \cdot \text{Ag} \right.$$
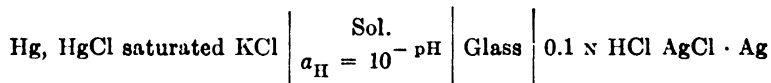
the E.M.F. is

$$E = \frac{RT}{NF} \text{pH} - e$$

[1] Since the publication of the preliminary report of this paper (Stadie and O'Brien, 1930), Fosbinder (1930) has described an electron tube circuit of the null type which employs one tube, the plate current of which is balanced out by a battery and resistances similar to the circuit of Rasmussen (1929) for the measurement of ionization currents. Our experience has been that it is difficult to eliminate the drifting of the zero of the galvanometer in one tube circuits although Fosbinder has been able to reduce it to 2 mm. per minute.

From which

$$e = \frac{RT}{NF} \, \text{pH} - E \qquad (1)$$

$$\frac{RT}{NF} = 0.0591 \text{ at } 25° = 0.0617 \text{ at } 38°$$

e, the cell constant, includes the potentials of the calomel and silver chloride half-cells as well as G, the glass potential of MacInnes. Since the system is used entirely as a reference system standardized against known phosphates, it is not necessary to select membranes with zero G potentials, nor need the silver chloride electrode or 0.1 N HCl be made with any great precision. It is well, however, to select electrodes with G potentials less than 2 millivolts. Our experience has been that electrodes whose G potentials are small have more stable electrical properties particularly with respect to constancy of e.

To determine e for a given glass cell at blood pH range it is sufficient to determine the E.M.F. of four or five phosphate buffers of known pH and to calculate e by Equation 1. With this value of e the pH of an unknown solution may be calculated from the equation.

$$\text{pH} = \frac{E + e}{\dfrac{RT}{NF}} \qquad (2)$$

In practice it is frequently found that a glass electrode does not yield theoretical values for $\dfrac{RT}{NF}$. Such an electrode need not be discarded. It is merely necessary to determine the empirical value of the constants, e and $\dfrac{RT}{NF}$, over the given pH range, with known buffers, and with these constants to calculate the pH of an unknown solution by Equation 2.

Constancy of e, the Cell Constant

Daily determinations of the mean value of e with six phosphate buffers over a period of days show a variability of e which reflects changes in the properties of the Ag·AgCl electrode, changes of the

concentration of HCl in the side arm due to absorption of alkali from the glass and action with AgCl, etc.

These changes, whatever their nature, are unimportant if they are sufficiently slow to allow the system, once having been standardized by buffers, to be used as a reference system of relatively great constancy over a period of a day or several days. From our experience we can say that a carefully selected electrode will show no changes (< 0.1 millivolt) of e over the course of a day and only slight changes from day to day. Table I is given as a typical example of the behavior of a good electrode with respect to these

TABLE I

Cell Constant of Glass Electrode Over a Period of Time

Date	e_0
1930	*volt*
Apr. 14, a.m.	0.1185
" 14, p.m.	0.1186
" 15.	0.1191
" 16.	0.1189
" 17, a.m.	0.1191
" 17, p.m.	0.1189
" 18.	0.1196
" 23.	0.1153
" 24.	0.1164
" 25.	0.1173
" 28.	0.1154
" 29.	0.1102
May 3.	0.1030

changes. From Table I it is apparent the drift of e is so slow that for any one day it is constant. On the other hand, some electrodes, particularly when new, behave in a more erratic fashion. It is wise then to determine e frequently until the character of the electrode is established.

pH of Phosphate Buffers and Reversibility to Temperature

The degree of accuracy in the measurement of the E.M.F. of a series of buffers which the glass electrode may be made to give is illustrated in the following experiment. Four phosphate buffers covering the range 6.8 to 7.8 were carefully prepared according to

the data of Hastings and Sendroy. The E.M.F. of each phosphate buffer was then determined by means of the glass electrode at 25° and 38°. These values (Table II) were plotted on a large scale against the pH and a straight line drawn through the points. In no case was the deviation of any point from the line more than 0.2 millivolt and the average deviations were 0.08 and 0.13 millivolt. At both temperatures the slopes of the lines were the theoretical ones, i.e. 0.0591 and 0.0617 volts per pH respectively. In other words, as a reference system for the determination of pH of such well buffered solutions as phosphates, the glass electrode is susceptible of an accuracy of at least 0.1 millivolt or 0.0016 pH unit and further it is reversible in respect to temperature.

TABLE II

Linear Relationship between E.M.F. of Glass Electrode and pH of Phosphate Buffers at 25° and 38°

Series 1. $t = 25.0° \pm 0.05°$, $e = 0.1007$ volt, E.M.F. (calculated) $= 0.0591$ pH $- e$				Series 2. $t = 38.0° \pm 0.05°$, $e = 0.1134$ volt, E.M.F. (calculated) $= 0.0617$ pH $- e$			
pH	E.M.F. observed	E.M.F. calculated	Δ	pH	E.M.F. observed	E.M.F. calculated	Δ
	volt	*volt*	*mv.*		*volt*	*volt*	*mv.*
6.958	0.3101	0.3100	+0.1	6.958	0.3136	0.3135	+0.1
7.261	0.3277	0.3278	−0.1	7.261	0.3320	0.3320	0.0
7.412	0.3367	0.3366	+0.1	7.412	0.3414	0.3416	−0.2
7.705	0.3545	0.4545	0.0	7.705	0.3587	0.3585	+0.2
Mean.....................			±0.08				±0.13

Determination of G, the Glass Potential

For the cell

$$\text{Hg, HgCl saturated KCl} \mid 0.1 \text{ N HCl AgCl, AgCl}$$
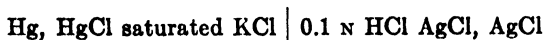
the E.M.F. is

$$E = RT \, \text{pH}_{HCl} - e$$

By the interposition of a glass membrane the cell

$$\text{Hg, HgCl saturated KCl} \mid 0.1 \text{ N HCl} \mid \text{glass} \mid 0.1 \text{ N HCl AgCl, Ag}$$
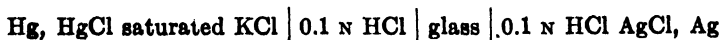
has an E.M.F.

$$E' = RT \; \text{pH}_{HCl} - e + G$$

where G is any E.M.F. due to the glass membrane itself. Accordingly we get

$$G = E - E'$$

To determine G we measure the E.M.F. of a silver chloride and a calomel half-cell combined as above with and without the interposition of a glass membrane.

Table III shows the distribution of the G values of a series of 89 unselected glass electrodes determined in the above way.

TABLE III

Asymmetry Potential of Unselected Glass Electrodes

Series 1, 5 mm. in diameter			Series 2, 3 mm. in diameter		
G	No. of specimens	Per cent of total	G	No. of specimens	Per cent of total
mv.			*mv.*		
0	2	4	0	6	14
0 −0.5	14	30	0 −0.5	2	5
0.5–2.0	10	21	0.5–2.0	3	7
2.0–5.0	13	28	2.0–5.0	4	10
5.0	8	17	5.0	27	64
	47			42	

These electrodes were kept for several days in 0.1 N HCl before the readings were made. If a value of $G = 2$ millivolts is selected as the outside limit for a good electrode, it is apparent from the table that the smaller good electrodes (24 per cent for small; 55 per cent for large) are scarcer in any given batch, but since the preparation and testing is so easy, plenty of good electrodes of either size may be obtained by a few hours preparation.

Diurnal Changes of Asymmetry Potential

We have referred above to slow changes in the value of e, the cell constant, which may be caused by a number of changes in the

electrode.   These may be enumerated in the order of their impor-
tance as (1) change of pH of the HCl in the side arm due to absorp-
tion of alkali from the soft glass, (2) evaporation of water from the
HCl, (3) reaction of HCl with O_2 and silver diminishing the con-
centration of Cl^-.

All these reactions are slow but may fully account for gradual
changes in e.   But in addition to these there may occur a more
or less sudden change of G, the asymmetry potential reflecting
itself in a change of e.   In Table IV we give the record of the G
values of a few electrodes determined from the day of manufacture.
The electrodes were kept in 0.1 N HCl between the determinations.
The point established by the data in the table is that some elec-
trodes show sudden and considerable changes in G and unless the

TABLE IV
Diurnal Changes in Asymmetry Potentials of Glass Electrodes

Specimen No.	Days				
	1	2	3	4	7
	mv.	*mv.*	*mv.*	*mv.*	*mv.*
1	0.6	0.5	0.5	1.9	
2	3.7	0.0	1.2	1.2	1.3
3	5.3	1.5	0.3	1.8	1.0
4	4.1	0.1	2.3	1.6	−0.8
5	3.0	2.5	1.9	1.1	−0.3

value for the cell constant which includes G is reestablished by at
least daily determinations with standard phosphates considerable
error may result in the calculation of pH.

Filling the Electrode

The blood or serum which of course must be handled by ap-
proved technique to prevent loss of CO_2 is contained either in a
mercury receiver or a small 2 cc. Luer syringe: The receiver or
the syringe is connected to the lower end of the electrode pipette
and the serum or blood slowly forced in until both bulbs of the
electrode are filled.   The function of the accessory bulb is now
apparent.   (1) The first portion of the serum or blood which
comes in contact with air in the electrode may lose a little CO_2 but
is forced up into the accessory bulb.   (2) It serves as a reservoir

for excess serum, a small part of which is used to establish the liquid junction.

Loss of CO_2 from Serum during Filling of Electrode

The fact that serious errors may occur in the determination of the pH of serum on account of loss of CO_2 during manipulation is of course commonplace to experienced workers and it is for this reason that particular attention has been given to the details of the technique which will eliminate this as a source of error. It is assumed that the serum has been collected and handled in proper fashion and is contained either in a mercury receiver or a small

TABLE V

Loss of CO_2 during Filling of Electrode with Serum, $t = 38°$

Large electrode increase of pH without CO_2	Small electrode change of pH without CO_2
0.007	0.003
0.008	0.002
0.014	−0.005
0.013	0.002
0.000	−0.006
0.002	0.000
0.003	−0.002
	+0.002
Mean........0.006	±0.002

Luer syringe. The question whether CO_2 loss occurs in transferring to the electrode where exposure to air occurs is answered by the following experiment. A serum equilibrated with 40 mm. of CO_2 was transferred to a mercury receiver and alternate determinations of pH were made. In one case the air in the pipette was first swept out by 100 cc. of a CO_2-air mixture containing 40 mm. of CO_2. In the second case this operation was omitted. The results are given as differences of pH with and without CO_2 in Table V both for a large and small electrode whose volumes (exclusive of the accessory bulb) were 0.5 and 0.2 cc. respectively. In the case of the large electrode, the surface exposed to air is relatively great and there is apparently a loss of CO_2 which is slight but regularly causes an increase which averages 0.006 pH over the control not exposed to air. In the case of the small electrode the

surface exposed is much less and there is no loss of CO_2 since the differences in pH with and without CO_2 are dispersed about zero and average ±0.002 pH. It is advisable then to use electrodes which have small volumes of 0.2 to 0.3 cc. so as to eliminate the necessity of sweeping out the electrode with a CO_2 mixture. The small electrodes possess excellent electrical properties and moreover conserve material.

Effect of Albolene, Alcohol, and Ether in Electrode

The use of albolene is common in serum collection to prevent loss of CO_2. We have found that its introduction into the electrode where it might come into contact with the membrane leads to a serious alteration of e, the cell constant, which can only be restored to its original value by prolonged washing of the membrane with water. It is necessary to avoid the introduction of even small amounts of albolene into the electrode by proper selection of technique. Drying of the electrode and membranes by alcohol and ether followed by aeration does not alter the value of e. If desired this technique may be used particularly in small electrodes to avoid dilution of serum by wash water or saline retained in the electrode.

Comparison of pH of Serum Determined with Glass Electrode and Hydrogen Electrode at 38°

The method used for determining the pH with the hydrogen electrode was a modification of Simms' (1923) method employing water-jacketed electrodes. The serum at 38° was equilibrated by bubbling through it a gas mixture of CO_2 (at 40 mm. of Hg) and hydrogen until two successive readings of the E.M.F. were constant. The serum was transferred to a small Luer syringe, with rigid precautions to prevent exposure of the serum to air, and then transferred from the syringe to the glass electrode which was also maintained at 38°. The E.M.F. readings of the serum in the glass electrode rapidly became constant and in all cases remained so for a half hour of observation. The results are given in Table VI. The difference between the two methods averages 0.007 pH unit. All of the glass electrode values are more alkaline probably on account of slight losses of CO_2 during transfer. On the whole, considering the sum of the error involved in collection, transfer, etc., the experiment shows that the glass electrode

TABLE VI

Comparison of pH of Serum Equilibrated with CO_2 at 40 Mm. by Hydrogen
and Glass Electrodes at 38°

Specimen No.	paH hydrogen electrode	paH glass electrode	ΔpH
1	7.420	7.431	+0.011
2	7.438	7.446	+0.008
3	7.437	7.463	+0.026
4	7.430	7.446	+0.016
5	7.438	7.455	+0.007
6	7.445	7.442	−0.005
7	7.444	7.447	+0.003
8	7.443	7.440	−0.003
9	7.441	7.439	−0.002
10	7.421	7.441	+0.020
11	7.470	7.470	0.000
12	7.410	7.415	+0.005
13	7.420	7.418	−0.002
14	7.415	7.420	+0.005
15	7.395	7.405	+0.010
16	7.462	7.472	+0.010
17	7.470	7.473	+0.003
18	7.410	7.413	+0.003
19	7.385	7.391	+0.006
20	7.360	7.370	+0.010
21	7.395	7.397	+0.002
22	7.360	7.358	−0.002
Average...			±0.007

TABLE VII

Reproducibility of pH Determination by Glass Electrode at 38° on Sample of
Equilibrated Beef Serum

Sample No.	paH	Δ from mean
1	7.360	0.000
2	7.363	0.003
3	7.357	−0.003
4	7.363	0.003
5	7.360	0.000
6	7.360	0.000
7	7.362	0.002
8	7.358	−0.002
9	7.358	−0.002
	7.360	±0.0016

might be considered to give pH values of serum to within somewhat less than 0.01 pH of the values of the hydrogen electrode.

To show the degree of reproducibility of pH readings on serum given by the glass electrode, Table VII gives a series of pH determinations on a sample of serum transferred from a mercury receiver to the electrode. The average deviation of the series from the mean is ±0.0016 pH unit. The error of the pH determination

TABLE VIII
Time of Equilibrium in Glass Electrode

Experiment 1			Experiment 2		
Elapsed time		E.M.F.	Elapsed time		E.M.F.
Whole blood at 38°					
min.	*sec.*	*volt*	*min.*	*sec.*	*volt*
	17	0.3461		20	0.3437
	47	0.3468		50	0.3452
1	17	0.3472	1	20	0.3452
5		0.3474	5		0.3453
10		0.3472	10		0.3456
40		0.3470	20		0.3455
				40	0.3452
Experiment 3			Experiment 4		
Phosphate buffer at 38°					
	16	0.3358		15	0.3362
	46	0.3363		45	0.3365
1	16	0.3365	2		0.3365
3		0.3365	3		0.3365
5		0.3365	5		0.3365
60		0.3365	60		0.3365

with the glass electrode is shown by this experiment to be quite small and probably negligible in comparison to other errors involved in the collection of blood.

Time Required for Equilibrium of Glass Electrode with Serum and Phosphates

The attainment of equilibrium of phosphate buffers, serum, or whole blood in the electrode is quite rapid and once established a

constant E.M.F. is maintained for hours, if proper precaution for the preservation of whole blood or serum is used. In the case of phosphates we were able to demonstrate equilibrium (within 0.3 millivolt) 11 seconds after the electrode was filled with the buffer solution. In the case of whole blood it is possible to demonstrate that equilibrium is reached in about 30 seconds. Table VIII gives the results of two experiments on whole blood and phosphates designed to show the time required for a constant E.M.F. reading in the glass electrode.

SUMMARY

1. A glass electrode suitable for the determination of the pH of serum at 38° is described.

2. A modification of Stadie's electron tube potentiometer is described which is very stable, shows practically no drifting of the zero, and has a sensitivity of 10 to 15 mm. of deflection per millivolt. It measures the absolute E.M.F. of glass electrodes to 0.1 millivolt without drawing appreciable current from the cell.

3. The behavior of glass cells of the MacInnes type, with respect to cell constant and asymmetry potential, is discussed.

4. Comparative pH determinations of serum by the hydrogen and glass electrodes are reported. An accuracy of <0.01 pH unit for serum handled with proper technique is obtainable.

5. The time required for blood and serum to give a constant E.M.F. equilibrium in the glass electrode is about 30 seconds and constant readings may be attained for hours.

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COMPOSITION OF BONE

X. MECHANISM OF HEALING IN LOW PHOSPHORUS RICKETS*

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The inorganic serum phosphorus[1] is usually low in active rickets. When the rickets heals, the phosphorus rises. Similarly in low calcium rickets, healing is accompanied by a rise in serum calcium. These observations were given a roughly quantitative form in the Ca × P product of Howland and Kramer (2). When healing of rickets occurs, resumption of calcification is usually accompanied by an increase in this empirical product. In this paper, experiments are reported which demonstrate that the rise in the Ca × P product precedes the resumption of calcification in the healing of low phosphorus rickets in rats.

Preliminary Investigation[2]

Experiment I—In all of these preliminary experiments, Ration 2965 of Steenbock and Black (3) was fed to rats until severe rickets developed. Then cod liver oil was incorporated in the food so that it constituted 0.5 per cent of the diet. The rats were autopsied at intervals after addition of the cod liver oil, and the tibias were examined immediately for new calcification, a 5 per cent AgNO3 solution being used to stain the bone deposits.

It was found that resumption of calcification occurred between the 3rd and 6th days after the administration of the curative agent.

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* Part of the data in this paper was presented before the Montreal meeting (1) of the American Pediatric Society, June 18, 1930.

[1] In this paper, the concentration of inorganic serum phosphorus will be referred to hereafter simply as phosphorus.

[2] Dr. Leo V. Rosenthal cooperated in the preliminary investigation.

Experiment II—In order to fix more closely the time of the first appearance of new calcification, the ricketic rats were autopsied at shorter intervals in the second experiment.

It was found that new calcification first appeared 3.2 days after the addition of the cod liver oil to the diet.

Experiment III—In order to correlate the change in phosphorus with the occurrence of new calcification, a set of forty-one ricketic rats was employed in the third experiment.

TABLE I

Large Amount of Cod Liver Oil, Rapid Healing

Rat No.	Time on cod liver oil	Ca	P	Ca × P	New calcification
	days	*mg. per 100 cc.*	*mg. per 100 cc.*		
58–61	0.0	12.1	2.4	29	—
62–66	3.0	10.5	5.1	54	++
67	3.0	10.0	5.1	51	—
68–70					++
72	3.7	10.3	5.0	52	+
71, 73, 74					++++
75–78	4.9	8.1	5.2	42	+++
79–82	5.8	11.9	5.4	64	++++
83–88	6.8	11.6	4.2	49	+++
89–94	7.9	12.3	5.4	66	++++

New calcification began to appear 3.5 days after cod liver oil had been added to the diet. However, there was a marked individual variation in the time of the first response to the antiricketic agent. Thus even after 5.5 days one of the rats showed no evidence of new calcification. No striking correlation between the appearance of new calcification and the phosphorus values was noted.

Experiment IV—In this and in all of the following experiments, analyses were made of serum calcium as well as of phosphorus in order to obtain the Ca × P product.

In Experiment IV, thirty-seven rats were used. The four rats autopsied at the start showed severe rickets with a low product of 29. The other rats were autopsied at intervals beginning 3 days after transfer to the curative diet. The data are given in Table I. It is seen that healing had started before the end of the 3rd day, by which time considerable amounts of new calcification were already present.

TABLE II

Spontaneous Healing

Rat No.	Time on cod liver oil	Ca	P	Ca × P	New calcification
	days	*mg. per 100 cc.*	*mg. per 100 cc.*		
95, 96, 98, 99	0.0	10.4			—
97					+
100–103	1.0	10.5	2.1	22	—
104, 105, 107	2.0	10.5	2.6	27	—
106					+
108, 109, 111	2 7	11.2	2.4	27	—
110					++
112–115	3.0	11.9	2.7	32	—
116, 117, 119	3.7	10.2	3.5	36	+
118					—
120–123	3.8	12.0	3.3	40	+
124	4.1	10.9	3.7	40	—
125–127					+
128–130	4.3	11.6	3.6	42	+
131–133					—

Inspection of the data on food consumption showed that the rats in Experiment IV had consumed about twice as much food *per diem* as had the rats in the previous experiments. With the increased food consumption, these rats had ingested larger amounts of cod liver oil daily. This most probably accounts for the earlier appearance of new calcification in the rats of this experiment.

With the presence of new calcification, the phosphorus was high; it had risen from 2.4 to 5.1 mg. per cent. Strangely enough, the calcium had dropped from 12.1 to 10.5 and continued to drop to the unexpectedly low level of 8.1. It subsequently rose to 12.3 mg. per cent. The calcium showed far more fluctuation than did the phosphorus in this period of adjustment.

Whenever new deposition was noted, the Ca × P product was above 40.

Experiment V—In this experiment thirty-nine ricketic rats were treated as shown in Table II.

It is seen that the Ca × P products rose steadily from 22 to 42 in the course of about 4 days. The curative diet did not produce calcification until 3.7 days had elapsed. In the meantime the product had steadily risen from 22 to 36.

In the second group, the calcium was 10.5, the phosphorus 2.1, and the product was 22; no new calcification was present. 2 days later, the calcium had risen to 11.9, and the phosphorus to 2.7; no new calcification was present. Thus the Ca × P product had risen from 22 to 32 before calcification was resumed.

Results of Preliminary Experiments

It is evident that there may be a definite fluctuation in the serum calcium in the 1st week following the administration of the curative diet. It has generally been assumed that only the phosphorus varies in the healing of low phosphorus rickets. There is also a marked individual variation as regards the time of response to the antiricketic agent.

It was obvious that, so far as determining the sequence of events in the healing of rickets was concerned, clear cut results were not obtained because of the pooling of the bloods before histological examination. When the mixed bloods were those of rats which gave similar histological findings as regards the presence or absence of new calcification, all was well. But when, as often happened, bloods were pooled from rats with widely different histological pictures, the chemical results were difficult to interpret.

Radiographic examination before autopsy does not serve to differentiate the rats in which healing has just started from those in which no healing is present, for the radiograph does not always reveal slight amounts of newly deposited bone. This observation

has also been made by Marshall and Knudson (4). The radiographic technique is not as delicate as the histologic for this particular purpose.

An unambiguous answer to the problem stated at the beginning of this paper requires the development of a technique of collecting the blood from each rat separately. The sera should be pooled for chemical analysis *after* the histological examinations have been made. Only those sera should be combined which are obtained from rats showing similar histological pictures.

Final Technique

Collection of Blood—The method finally adopted is seen by referring to Fig. 1. A small test-tube 7.5 cm. long and 1 cm. in diameter is supported in a 15 × 2 cm. test-tube by means of some cotton packing placed at the bottom of the large test-tube. The height of the packing is so adjusted that a stemless funnel, 4 cm. in diameter, when held supported on the mouth of the large test-tube reaches a few mm. below the mouth of the small test-tube. The rat is killed by cutting the carotid arteries, without anesthesia, and the blood is collected in the small dry test-tube. A different test-tube is used for each rat. The blood is allowed to clot. The clot is rimmed with a thin dry glass rod and the tube is centrifuged. The serum is then separated from the clot.

The tibias are removed and examined histologically immediately after death. The sera of rats showing the same amount of new calcification are then combined and analyzed. The sera of rats showing no new calcification are also combined and analyzed. The sera from rats showing spontaneous healing are discarded. The confusion which resulted when bloods were pooled before histological examination is thus avoided.

This procedure of collecting separately the blood from each rat was followed in all of the following experiments.

Histological Examination—The tibias were examined as soon as the rats were killed. Each tibia was cut into at least four and preferably into five sections. All of these sections were stained at once with 5 per cent AgNO$_3$ solution and examined with the binocular microscope. In the very early stages of healing, it frequently happened that some of the sections of a given tibia showed no trace whatsoever of new calcification, while other sections of the same

tibia showed fragments of a line, or even a complete line, of new deposit at the provisional zone of calcification.

When healing starts, calcification is not resumed simultaneously at the provisional zone throughout the tibia; most often the new calcification first appears at the periphery of the tibia. The "end" sections often show fragments of a line, or even a complete line, of new calcification at the provisional zone even when sections cut from the middle of the same tibia showed no trace of new calcification. Sometimes this was reversed; the "end" sections of a given tibia showed no healing, while the middle sections showed new calcification at the provisional zone.
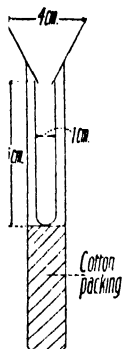
Fig. 1. Apparatus, consisting of a small test-tube, a stemless funnel, and a support, employed for the collection of blood.

Therefore, at least four sections of each tibia were examined before it was concluded that new calcification was present or absent.[3]

Spontaneous Healing—Occasionally one or more rats will show spontaneous healing, *i.e.* unexpected healing due to some unknown factor.[4]   In some laboratories radiograms are taken of all the supposedly ricketic rats at the beginning of an experiment in order

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[3] One tibia of each rat was used for this histological examination.   The other tibia was decalcified, embedded in celloidin, sectioned, and stained with eosin-hematoxylin.   These latter sections will be discussed in subsequent papers of this series (unpublished data).

[4] Fasting produces a rise in phosphorus with consequent healing of the rickets; therefore, rats which lost weight during the experimental period were rejected.

to determine whether any of the rats should be discarded because of spontaneous healing. However, very early healing may sometimes escape detection by this method. Furthermore, healing within the metaphysis, or at the head of the shaft, is frequently undetectable by means of x-ray examination.

At autopsy, the histological examination reveals the presence of spontaneous healing and also the approximate age of the new calcification.

TABLE III

Healing Induced by Cod Liver Oil

Rat No.	Time on cod liver oil	Ca	P	Ca × P	New calcification
	days	*mg. per 100 cc.*	*mg. per 100 cc.*		
135–138	0.0	10.7	2.5	27	—
139–142	2.8	11.7	2.9	34	—
143, 145, 146, 148, 149	3.6	12.9	3.2	41	—
153, 154, 156–158	3.9	12.7	4.0	51	—
162, 164, 165, 169–171	4.2	11.7	3.2	37	—
144, 147	3.6	12.3 ⎱	3.9	47	+
150–152, 155, 159	3.9	11.9 ⎰			+
160, 161, 163, 167	4.2	11.6	4.0	46	++
166					+++

Final Experiments

Effect of Added Cod Liver Oil (Steenbock Diet)—In this experiment rickets was produced by means of Ration 2965. (In all of these experiments the rats were given the rickets-producing rations when 28 days old; severe rickets was invariably produced in 21 days.) The thirty-six ricketic rats were then given a curative diet consisting of Ration 2965 plus 0.5 per cent cod liver oil. The rats were autopsied as shown in Table III. The blood of each rat was collected separately and the sera were mixed after histological examination as described in the previous section.

It will be seen from Table III that the initial Ca was 10.7 and the initial P was 2.5 mg. per cent; the initial product was 27.   Following ingestion of cod liver oil the calcium rose as high as 12.7 and the P as high as 4.0 and yet no new calcification was apparent. *Thus the Ca × P product had risen from 27 to 51 prior to the appearance of new calcification.*

Here we had a clear cut, unambiguous correlation between the histological picture on the one hand and the serum Ca and P on the other.   The rats had been observed from a period before any calcification could be present, through the appearance of the very first traces of new calcification, to the development of a considerable amount of new deposit.   The Ca × P product increased markedly *before* calcification appeared.

Effect of Added Cod Liver Oil (Hess's Diet)—In the foregoing experiment, the rickets had been produced by means of Ration 2965.   Recently, Hess, Weinstock, Rivkin, and Gross (5) reported the production of a type of rickets in which the Ca × P product was above rather than below normal in many instances.   They stated that this type of rickets was extraordinarily resistant to treatment with specific antirickctic measures.   Their diet differed from Ration 2965 only in that yellow corn-meal was substituted for the ground, whole yellow corn.

In this experiment, thirty-seven rats were made rickelic by feeding a diet made up in this way.   Then the same cod liver oil previously used was incorporated in this new diet so that it constituted 0.5 per cent of the ration, as had been done with Steenbock's diet.   The rats were then treated as shown in Table IV.

The rickets produced by the new diet was very severe, as might have been expected.   The calcium in the initial group was 10.2 and the phosphorus 1.5 mg. per cent.   The Ca × P product was extremely low, *i. e.* only 15.

1 day after cod liver oil had been added to this diet, the phosphorus rose to 2.2, raising the Ca × P product from 15 to 23.   No new calcification was evident.   After 2.8 days on the curative diet, the Ca × P product was 25.   No new calcification was yet demonstrable.   This picture was maintained unchanged for the next 24 hours.   New calcification was first demonstrated 2 days later, 5.8 days after cod liver oil had been added to the diet.

At this time some of the rats showed beginning healing, whereas

an approximately equal number showed no healing whatsoever. The sera were pooled as shown in Table IV. Those that showed no new calcification whatsoever had a calcium of 11.7 and a phosphorus of 2.3 mg. per cent. Both of these values were definitely greater than the initial values. *The Ca × P product had therefore risen steadily from 15 to 27 and yet no new calcification had appeared.* In the group in which calcification had been resumed the product was 47. The next day, the remaining rats were killed. These showed more advanced healing; the Ca × P product was 48.

TABLE IV

Healing Induced by Cod Liver Oil

Diet of Hess *et al.*

Rat No.	Time on cod liver oil	Ca	P	Ca × P	New calcification
	days	*mg. per 100 cc.*	*mg. per 100 cc.*		
172–175	0.0	10.2	1.5	15	—
176–180	1.0	10.4	2.2	23	—
181–185	2.8	11.4	2.2	25	—
186, 187, 189–191	3.8	10.8	2.3	25	—
195–197, 199–202	5.8	11.7	2.3	27	—
188, 198	5.8	12.3	3.8	47	+
192–194					++
203–208	6.8	12.5	3.8	·48	+++

The healing of the rickets produced by this more severe diet therefore appears to be due to the same mechanism which operates in the healing of the rickets produced by Ration 2965 (compare Tables III and IV).

• The rickets which we obtained by means of this diet was accompanied by a Ca × P product *lower* than that obtained with Steenbock's ration. Furthermore, this type of rickets responded to curative treatment almost as rapidly as did the rickets produced by the standard diet: 0.5 per cent cod liver oil added to Hess's diet induced healing before the 6th day, in our exeriments; whereas in

the experiments reported by Hess even 40 times the curative dose of cod liver oil failed to initiate healing in 9 days.

These widely divergent results may be due to possible differences in the quality of the yellow corn-meal used in the different laboratories. Hess and his coworkers reported a Ca : P ratio of 11.5 : 1 for their diet. Analysis of the diet made up in our laboratory gave a Ca:P ratio of 6.1:1 for Hess's diet. Our data are given in Table V.

We had used Quaker yellow corn-meal and obtained a ratio of 6.1:1. We subsequently made up a batch of diet using Lily White corn-meal purchased from the same firm from which Hess had obtained his supply. On analysis of this diet we obtained results essentially the same as those obtained for the usual Steenbock Ration 2965 (see Table V).

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TABLE V

Analyses of Rickets-Producing Diets

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Diet	Ca	P	Ca:P
	per cent	*per cent*	
Steenbock and Black Ration 2965..	1.20	0.28	4.3:1
Hess *et al.* Quaker corn-meal.......	1.09	0.18	6.1:1
" " " Lily White corn-meal...	1.21	0.26	4.7:1

The values we obtained for Ration 2965 were in agreement with the analyses of this ration made by other investigators. Our analysis of Hess's diet made up with Quaker yellow corn-meal showed a phosphorus content lower than that of Steenbock's ration, but not as low as 0.128 the value which Hess reported for his diet. With the Lily White yellow corn-meal diet we found much more than the 0.128 per cent phosphorus reported by Hess and his coworkers.[5]

Effect of Added Viosterol—Rickets was produced in a set of

[5] Dr. D. H. Shelling of Johns Hopkins University informs us that he was never able to obtain values for phosphorus as low as 0.128 per cent. He analyzed a number of diets made up according to the directions of Hess *et al.* (5), using a number of specimens of yellow corn-meal bought in Baltimore. His analyses yielded values ranging between 0.24 and 0.26 per cent for the phosphorus content of such diets.

thirty-one rats by Ration 2965 as usual.   Then viosterol,[6] diluted
with olive oil, was added to the diet so that its vitamin D content
was equivalent to the diet containing 0.5 per cent cod liver oil.

In the initial group of rickets rats, the calcium was 10.9, the
phosphorus was 1.9, and the product was 21.   The other groups
were autopsied as shown in Table VI.

It is seen that healing first appeared after 3 days, and that the
product rose *before* new calcification appeared.   The calcium rose

TABLE VI

Healing Induced by Viosterol

Rat No.	Time on viosterol	Ca	P	Ca × P	New calcification
	days	*mg. per 100 cc.*	*mg. per 100 cc.*		
320–322	0.0	10.9	1.9	21	−
323–326	2.0	11.1	3.0	33	−
327–330	2.4	10.9	3.0	33	−
331, 332, 334 336 337 339	3.0 3.1 3.2 3.3	13.3	2.9	39	−
342–347, 349	3.4	12.2	2.8	34	−
333 335 338 340, 341	3.0 3.1 3.2 3.3	13.5	3.6	49	+ ++ ++ ++
348 350	3.4		4.2		+ ++

from 10.9 to 13.3; the phosphorus rose from 1.9 to 2.9; the Ca × P
product rose from 21 to 39 and yet no new calcification had ap-
peared.   When healing did occur the product was 49.

The last two rats gave only enough serum for a phosphorus
determination; the value was 4.2 mg. per cent.

Thus the results obtained with viosterol are completely anal-
ogous to those obtained with cod liver oil.

Effect of Added Phosphate—The results obtained in the preceding experiments pointed to the increase in the Ca × P product as the determining factor in the resumption of calcification. Following administration of the curative diets, the Ca × P product rose steadily before new calcification made its appearance. In no case was calcification resumed before the Ca × P product reached a value of 40.

Since in these experiments 3 days or more elapsed following the administration of cod liver oil, or of viosterol, before new calcification appeared, there was the possibility that changes due to vitamin D might have been going on preparatory to calcification. Such possible changes might have been responsible for the deposi-

TABLE VII

Healing Induced by Phosphate

Rat No.	Time on phosphate	Ca	P	Ca × P	New calcification
	days	mg. per 100 cc.	mg. per 100 cc.		
209–211	0.0	10.4	2.5	26	–
212–215	1.1	7.9	8.0	63	++
216–219	1.2	8.3	8.7	72	++
220–223	2.0	5.9	8.9	53	++
224–227	4.0	4.1	9.6	39	++
228–231	5.9	4.8	10.1	49	++++
232–239	8.0	5.3	8.5	45	++++
240–246	11.9	5.2	12.0	62	++++

tion of new calcification. The question therefore arose as to whether the relation of new calcification to the Ca × P product was merely a coincidence, or whether the rise in the Ca × P product was the causative factor in the resumption of calcification.

To determine whether mere rise in the Ca × P product, without the intermediary intervention of vitamin D, would give similar results, thirty-eight rats were placed on Ration 2965 as in the earlier experiments. After rickets had developed, phosphate[7] was added to the ration. An equimolar mixture. of KH_2PO_4 and Na_2HPO_4 was used. Enough was added to the diet to change

[7] This ration differed but little from the analogous ration of Karelitz and Shohl (6) by which they obtained rapid healing of rickets in rats.

the Ca:P ratio from 4:1 to 1:1. The rats were then examined as summarized in Table VII.

It is seen that new calcification appeared in less than 26 hours after adding phosphate to the diet; and in 26 hours the Ca × P product had risen from 26 to 63. There had been a rapid rise in phosphorus. The rise was so rapid as to depress the calcium to 7.9 mg. per cent. With the continued ingestion of the changed ration, the serum phosphorus continued to rise to 9.6 in the fifth group with a concomitant drop in calcium to 4.1 mg. per cent. Many of these rats on being bled to death exhibited the muscular spasms characteristic of tetany.

TABLE VIII

Healing Induced by Phosphate

Rat No.	Time on phosphate	Ca	P	Ca × P	New calcification
	hrs.	*mg. per 100 cc.*	*mg. per 100 cc.*		
247–249	0	9.9	2.3	23	–
251–253, 255	6	8.7	4.1	36	–
256, 258, 260	11				–
264, 265	12	9.2	5.5	51	–
257, 263, 266	12	7.9	5.0	40	+
254	6	7.1	7.0	50	++
259, 261, 262	11				++

After 4 days on this diet, there seemed to be some adjustment on the part of the mechanism that regulates the level of the serum calcium for although the phosphorus continued to rise to 12.0, the calcium did not continue falling, but on the contrary rose to a value of 5.2.

New calcification was obtained more quickly than in the previous experiments in which vitamin D had been administered. New calcification had appeared with astonishing rapidity. It was also significant that this was paralleled by an equally rapid rise in the Ca × P product.

In the preceding experiment, resumption of calcification had occurred with such unexpected rapidity that it was impossible to

conclude whether the appearance of the new calcification preceded or followed the rise in the Ca × P product. In this experiment, therefore, the rats were autopsied during the first 12 hours after the addition of phosphate to the diet, as shown in Table VIII.

6 hours after putting the phosphate diet in the rat cages, five rats were autopsied. Rat 254 showed new calcification; the other four rats showed that *the Ca × P product had risen in 6 hours from 23 to 36 without the appearance of any new calcification.* The third group shows that within 12 hours the Ca × P product rose to a high value of 51 and yet no new calcification was observed. In those groups in which calcification was noted, the product was 40 or more.

Thus the rise in the Ca × P product preceded the appearance

TABLE IX

Healing Induced by Fasting

Rat No.	Time fasted	Ca	P	Ca × P	New calcification
	hrs.	mg. per 100 cc.	mg. per 100 cc.		
648–653	0	10.0	2.4	24	—
661–664	4	10.0	3.0	30	—
670–677	8	9.7	4.3	42	—
678–683	10	9.5	4.7	45	—
684–688	12	9.1	5.5	50	—
690–695	21	5.7	10.2	58	+

of new calcification whether healing was induced by means of cod liver oil, by viosterol, or by phosphate.

Effect of Fasting—Wilder (7) and Cavins (8) have confirmed the original observations of McCollum *et al.* (9) and of Kramer and Howland (10) that fasting cures rickets and that the serum phosphorus rises rapidly as a result of fasting. Table IX summarizes an experiment in which healing was induced by fasting.

A group of thirty-five rats made ricketic by Ration 2965 were placed in cages which contained water but no food whatsoever. Rats were autopsied during the first 24 hours of fasting as shown in Table IX. It is seen that, as a result of fasting, the phosphorus rose from 2.4 to 10.2 mg. per cent. There was a concomitant depression of the calcium from 10.0 to 5.7 mg. per cent; the Ca × P product rose steadily from 24 to 58. It is also seen that this prod-

uct rose in 12 hours to a high value of 50 *prior* to the appearance of new calcification. When calcification was noted at 21 hours, the product was 58.

Thus whether the healing of rickets be induced by cod liver oil, by viosterol, by phosphate, or by fasting, the rise in the product precedes the resumption of calcification.

Miscellaneous Experiments

Effect of Phosphate plus Cod Liver Oil—When phosphate was added to the diet the sudden increase in the serum phosphorus had resulted in a marked reduction in the serum calcium. The question arose as to whether cod liver oil might be able to prevent this reduction if it were given simultaneously with the phosphate.

In this experiment, twenty rats were made ricketic by Ration 2965 as usual. Then cod liver oil and phosphate were both added to the ration. The cod liver oil constituted 0.5 per cent of the diet and the Ca:P ratio was made 1:1 by the addition of an equimolecular mixture of Na_2HPO_4 and KH_2PO_4. The results are summarized in Table X.

At the start, the calcium was normal, the phosphorus was low, and all the rats showed severe rickets. The rats were then given the diet which contained cod liver oil and added phosphate. After 1 day on this diet, the phosphorus had risen to 6.3, and the Ca × P product had risen to 60; beginning healing was evident in all five rats. However, two of the rats on being bled developed the convulsions characteristic of tetany, showing that the cod liver oil had not been able to prevent the ill effects of the sudden rise in the phosphorus.

The diet was therefore changed to one in which only cod liver oil was added to Ration 2965 in the same proportion as before. The new diet contained no added phosphate.

The next day the third group was killed. The calcium was found to have risen to 11.1; the phosphorus however, had *dropped* to its original low level. The Ca × P product had dropped from 60 to 22. The extent of the healing showed no increase over that found in the preceding group.

The last group was killed 3 days later. All the rats showed recurrent rickets. The 4 day old line of new calcification was not at the provisional zone, but was within the metaphysis. A new

provisional zone had appeared above this line showing that healing had ceased and that rickets had again developed. In three of the rats (Rats 287, 288, and 290) there was evidence of recurrent healing; at the provisional zone there were the beginnings of a new line.

Apparently healing had started in all the rats by the end of the 1st day because the added phosphate in the diet had increased the Ca × P product to 60. When the phosphate was removed from the diet the product dropped to 22, healing stopped, and the rickets recurred. By the 5th day the more slowly acting cod liver oil had,

TABLE X

Effect of Phosphate + Cod Liver Oil

Rat No.	Time on cod liver oil	Time on phosphate	Ca	P	Ca × P	Remarks
	days	*days*	*mg. per 100 cc.*	*mg. per 100 cc.*		
272–276	0.0	0.0	9.9	1.8	18	Severe rickets
277–281	1.0	1.0	9.5	6.3	60	Beginning healing
282–286	1.9	*	11.1	2.0	22	No further healing
289, 291 287, 288, 290	4.8	*	12.5	3.0	38	Recurrent rickets " " with recurrent healing

* At the end of the 1st day the diet was changed to one which contained 0.5 per cent cod liver oil but no added phosphate.

without the assistance of added phosphate, succeeded in raising the product, whereupon healing was resumed.

It is particularly noteworthy that although cod liver oil may produce a marked rise in serum phosphorus 3 days after its addition to a low phosphorus diet, it could not prevent the serum phosphorus from dropping in 1 day from 6.3 to 2.0 mg. per cent. Apparently the mechanism by which cod liver oil raises the serum phosphorus and maintains it at a high level requires at least 3 days for its development.[8]

[8] *Cf.* the interesting experiments of Shohl, Bennett, and Weed (11) and of Shohl and Brown (12).

Effect of Radiation—In an experiment designed to test the anti-ricketic potency of the new General Electric Company's Sunlamp, rickets was produced in nineteen rats by means of Ration 2965. The initial control rats showed marked rickets; the Ca × P product was 26. The remaining rats were then exposed to the Sunlamp at a distance of 36 inches.

It was found that seven exposures of 15 minutes each at this distance induced healing; seven exposures of 5 minutes each under identical conditions were not sufficient to initiate healing. When twelve 5 minute exposures were given, one rat showed healing while the other two did not. Thus, at 36 inches, 5 minute exposures did not give good results, whereas 15 minute exposures give satisfactorily rapid healing.

TABLE XI

Ca × P Product in Active Rickets

Ca:P ratio in diet	New calcification	Serum Ca	Serum P	Ca × P
		mg. per 100 cc.	*mg. per 100 cc.*	
4.3:1	None	12.1	2.4	29
	"	10.5	2.1	22
	"	10.7	2.5	27
	"	10.4	2.5	26
	"	9.9	2.3	23
	"	9.9	1.8	18
	"	10.9	1.9	21
	"	10.0	2.4	24
6.1:1	"	10.2	1.5	15
17.5:1	"	12.1	2.5	30

Four exposures of 30 minutes each over a period of 5 days induced healing in one rat, while a second rat showed no healing.

The purpose of this experiment was to block out roughly the number of exposures required to induce healing with different exposure periods when the distance from the Sunlamp was 36 inches. The serum calcium and phosphorus value were only incidental; however, the correlation between the blood chemistry and the resumption of calcification was similar to that usually obtained in this laboratory.

Effect of Added Calcium—In the experiment summarized in Table IV, rickets had been produced on a diet made up as described by

Hess and his coworkers (5).   They reported that the rickets which they obtained with this diet was accompanied by a Ca $\times$ P product above rather than below normal.   The rickets which we obtained in that experiment was accompanied by a Ca $\times$ P product even lower than that ordinarily obtained by Steenbock's ration.

However, our analysis of such a diet gave a Ca:P ratio of 6.1:1 whereas Hess obtained for his diet a ratio of 11.5:1.   In order to obtain the high ratios reported by Hess, it was found necessary to add excess $CaCO_3$ to the diet. $CaCO_3$ was therefore added in the present experiment in sufficient excess to obtain a Ca:P ratio of approximately 17.5:1.

Rats fed this diet for 23 days all showed rickets at autopsy. The calcium of the pooled sera was 12.1 and the phosphorus was 2.5 mg. per cent.   The Ca $\times$ P was 30, a value which is below normal.

These results are given in the last line in Table XI.   This table also contains a summary of the initial groups of the various experiments in this investigation.   It is seen that active rickets was invariably accompanied by a Ca $\times$ P product of 30 or less.

CONCLUSIONS

The relation of the Ca $\times$ P product to the presence of new calcification in the healing of active rickets has been so regular as to suggest strongly a causal connection.   The failure of calcification with low products, and its occurrence with high products, has been noted not only in infantile rickets, but also in experimental rickets in rats.   That this same relationship holds true for calcification *in vitro* has recently been confirmed and extended by Hörste (13).

It has usually been found both in experimental rickets and in *uncomplicated* infantile rickets that when the Ca $\times$ P product is 30 or less, active rickets is present, and that when it is 40 or more, the rickets usually is healing.   In the present investigation, in which more than 350 ricketic rats were examined, this correlation was again confirmed and several additional points were brought to light.   In the first place, resumption of calcification was never observed to precede the rise in the product.   Whenever renewal of calcification was noted, the product had already risen above the ricketic level.   The data are summarized in Table XII.

The administration of the various curative agents caused the product to rise steadily from values below 30 to values as high as 51 prior to the appearance of new calcification. When ricketic rats were fasted, the product rose to high levels within 12 hours; healing was noted several hours later. *This state of affairs, a normal product with no endochondral calcification, persisted for only a few hours, for whenever the product rose above the ricketic level, calcification rapidly followed.*

The calcification obtained with added phosphate *in vivo* parallels the calcification that is obtained *in vitro*. When sections of ricketic tibias are immersed in inorganic serum solutions with Ca × P products of 30 or less, calcification is not obtained. However, when the product in such solutions is 40 or more calcification

TABLE XII

Sequence of Ca × P Product and Resumption of Calcification in Healing Rickets

Treatment	Ca × P before treatment	Ca × P during treatment	
		Highest value prior to healing	Lowest value with healing
Cod liver oil....................	27	51	46
" " "	15	27	47
Viosterol.......................	21	39	49
Phosphate......................	23	51	40
Fasting........................	24	50	· 58

is regularly obtained *in vitro* in about 9 hours (14). In this investigation, calcification was obtained *in vivo* in 6 to 12 hours after the phosphate was offered to the animals.

Of the various theories advanced to account for calcification (15), the precipitation theory offers the best explanation at the present time for these findings.

SUMMARY

1. Severe low phosphorus rickets was produced in rats by diets of various Ca:P content. The Ca × P product in the serum was always 30 or less during the cessation of calcification.

2. Healing, induced by cod liver oil or by viosterol, was noted histologically by about the 3rd day. The rise in the Ca × P product *preceded* the appearance of new calcification.

3. Healing, induced by fasting, was noted histologically within 24 hours. The rise in the Ca × P product *preceded* the appearance of new calcification.

4. Healing, induced by phosphate added to the diet, was noted histologically within 12 hours. Here, too, the rise in the Ca × P product *preceded* the appearance of new calcification.

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COMPOSITION OF BONE

XI. BINDING OF CALCIUM IONS BY SERUM

By M. J. SHEAR and M. M. OFFNER

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For the formation of bone, an adequate concentration of calcium ions in blood serum is required. Thus, Shipley, Kramer, and Howland (1) found that when the calcium was present as chloride, acetate, or lactate, calcification *in vitro* was readily obtained, but that when the same concentration of calcium was present as citrate, calcification was not obtained. Conductivity studies (2, 3) from this laboratory showed that the presence of citrate causes a marked diminution in the calcium ion concentration.[1] It is thus seen that when citrate was present, although the total calcium concentration was normal, calcification was not obtained because the citrate had lowered the calcium *ion* concentration.

There are still marked differences of opinion as to the forms in which calcium exists in serum, and as to the relative concentrations of these various forms. However, most investigators agree that in general only about half of the total serum calcium is present as calcium ion.

The evidence for the existence of unionized calcium complexes in serum has been drawn largely from ultrafiltration and dialysis experiments. Recently, an entirely independent line of study has provided additional evidence in support of this theory. Equilibration experiments (5, 6) showed that $CaHPO_4$ is considerably more soluble in serum than in corresponding inorganic serum solutions. This indicates that a large part of the calcium of serum is in an unionized form.

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[1] These qualitative findings have recently been substantiated by Wiley (4). He determined the degree of dissociation of calcium citrate and found that in a 0.0025 N solution about 75 per cent is in the unionized form.

The present paper is a report of experiments designed to obtain information, by means of conductivity titrations, on the binding of calcium ions by serum.

Procedures

Removal of Calcium from Serum—For the conductivity titrations it was desirable to remove the greater part of the calcium from the serum, and at the same time to alter the other constituents as little as possible. Loeb (7), in preparing solutions of serum globulin, noted some denaturation of the protein on dialyzing it against physiological saline made up in 0.001 N HCl. We also obtained varying degrees of denaturation, depending upon the type of manipulation employed. Various methods were tried with unsatisfactory results. Simple dialysis against NaCl solution was the method finally adopted.

At first, 0.9 per cent NaCl was used. It was found that the volume of the serum solution increased to an inconvenient extent and that too long a time was required to remove the serum calcium. Better results were obtained by dialyzing against 3 per cent NaCl. It was found that changing the outer solution once daily for 5 days resulted in removal of about 90 per cent of the serum calcium. Complete removal of the calcium by more protracted dialysis was deemed inadvisable in the light of the results obtained by Loeb and Nichols (8). These authors found that sera made Ca-free by protracted dialysis in 0.8 per cent NaCl suffered some diminution in their Ca-binding capacity. They suggested that a partial denaturation of the proteins may have occurred as a result of the prolonged dialysis. We therefore did not attempt to remove the calcium completely. The serum preparations used for the conductivity titrations contained about 0.5 mg. of Ca per 100 cc.

Removal of NaCl—The specific conductances of serum, and of the serum solutions prepared by dialyzing serum against NaCl, were much higher than was desired for the conductivity titrations. Further dialysis of the serum solutions against water removed a part of the NaCl; but as the salt content of the dialyzing serum solutions decreased, the proteins began to precipitate. Even after considerable protein precipitate had formed the conductance was still too high.

Burk and Greenberg (9) recently utilized urea to prepare protein solutions without the use of electrolytes. They dissolved casein in water containing 40 gm. of urea per 100 cc., and employed these solutions for osmotic pressure determinations. Adopting this useful property of urea, we dialyzed serum against water containing 15 gm. of urea per 100 cc. By the end of the 1st day 50 per cent of the serum calcium had been removed. After the 3rd day of dialysis the solution still contained 50 per cent of the original calcium. Apparently, the combined calcium could not be removed by dialysis because there was no other cation present to replace it.[2]

Therefore NaCl and urea were both employed. Serum was dialyzed for about 5 days against 3 per cent NaCl to remove most of the calcium; then the dialysis was continued for about 6 days against 15 per cent aqueous urea to remove the NaCl. By the latter treatment enough electrolyte was removed to reduce the conductance to the desired range. The dialysis was further continued for several hours against distilled water to remove some of the urea. This dialysis served to reduce the NaCl content still further. The solution was carefully watched during the dialysis against water, and when protein began to precipitate as a result of the diminution of the urea content, the dialysis was

[2] Loeb (10) found that when serum is dialyzed against distilled water at pH 7.4, from 25 to 45 per cent of the Ca remained inside the sac; whereas, when the dialysis was performed against 0.6 per cent NaCl at the same pH, all of the Ca was removed by dialysis. He suggested that, in the former instance, precipitation of Ca proteinate prevented some of the Ca from diffusing outward. Loeb and Nichols (11), in a series of interesting dialysis experiments, found that the ratio of [Ca] in the serum to [Ca] in the outside solution increases with decreasing NaCl concentration. They suggested that this fact also is probably the result of precipitation of Ca proteinate.

In view of the results obtained in the urea dialysis experiment, the question arises as to whether the bound Ca requires Na^+ to free it from its combination and thus render it dialyzable. If such should be the case, then a decrease in NaCl concentration may cause a reduction in the diffusible Ca, because there is insufficient Na^+ to liberate all of the bound Ca; and the lower the NaCl content the less Ca will be removable by dialysis. Such a mechanism may operate either alone, or in conjunction with precipitation of Ca proteinate, to prevent complete removal of the serum Ca in dialysis against solutions containing inadequate concentrations of salt.

stopped. The small amount of precipitated protein redissolved later during the reduction in volume, with consequent increase in urea concentration, produced by evaporation.

Concentration—The solutions were analyzed for calcium, total nitrogen, and non-protein nitrogen. If the protein content was too low, concentration was effected by evaporation. The collodion bags containing the serum solutions were exposed to the air at room temperature. Concentration by ultrafiltration and by centrifuging (12) was also attempted, but the results obtained were not as satisfactory. By evaporation the concentration could be increased to any desired extent. In a number of instances enough water was removed to produce solid serum that could be cut into cubes. These cubes were a transparent yellow in the case of unhemolyzed serum and a clear red in the case of hemolyzed serum.

Preservatives—Thymol, chloroform, and toluene were discarded as unsatisfactory, for they caused precipitation of some constituent of the serum. Phenol was found satisfactory. Before dialysis, enough of a 1 per cent solution was added to both the serum and the external solution to make the phenol concentration 0.15 per cent. Phenol in 0.1 per cent concentration was found to be insufficient to prevent bacterial growth. Concentrations greater than 0.2 per cent resulted in precipitation.

Methods and Apparatus—Calcium was determined by the Kramer-Tisdall method, employing aliquots of 2 cc. for serum and 10 cc. for the serum solutions. Because of the high urea content, the final solutions were diluted 1:25 for analysis of total nitrogen and non-protein nitrogen, which were determined by the gasometric method of Van Slyke (13).

The apparatus and titration methods employed in these titrations have been described in previous communications (2, 3). Unless otherwise stated, the volume of the initial solution was 20 cc. in each case.

The stock calcium chloride solution was made from $CaCl_2$. The calcium content of the solution was checked by analysis. The various dilute calcium solutions were adjusted to pH 7.4 on being made up to volume.

Sera—After preliminary studies with gelatin solutions, casein solutions, and sheep serum, human serum was used. The blood was obtained from cardiacs and nephritics by phlebotomy.

Calculations—The calculated minimum conductances of the various mixtures of $CaCl_2$ and serum solution were obtained as in previous titrations (2, 3), according to the simple rule of mixtures.

$$\text{Calculated minimum specific conductance} = \frac{v_1 c_1 + v_2 c_2}{v_1 + v_2}$$

where v_1 and v_2 are the volumes of the original solutions used in each particular mixture, and c_1 and c_2 are the respective specific conductances of the original solutions. The curves labeled "calculated minimum" were drawn through points obtained in this way.

Although in this paper no reference is made to calculated maximum curves, in previous papers (2, 3) such curves were used. Those calculated maximum curves were based on data obtained by means of dilution titrations with water. Those calculations needlessly involved so called corrected specific conductances. It may be pointed out here that simple addition of the observed specific conductances from each pair of dilution titrations will give the desired calculated maximum conductance. Thus, at any given total volume, the calculated maximum will be given by

$$\begin{array}{l}\text{Calculated maximum} \\ \text{specific conductance}\end{array} = \begin{array}{l}\text{(observed specific} \\ \text{conductance)}_{Ca\,solution}\end{array} + \begin{array}{l}\text{(observed specific} \\ \text{conductance)}_{protein\,solution}\end{array}$$

where the observed values are those observed at that volume in the respective dilution titrations.

Both in this paper and in an earlier one (3), specific conductances were used in the calculations; whereas, in the first paper (2), resistances were used. The earlier method of calculation, using resistances, has been discontinued. Although it gave values which approximated those obtained by the formulas used subsequently, the later formulas, using conductances, are preferred on theoretical grounds.

Titration Curves

Protein Solutions—Solutions containing 1 and 5 per cent gelatin were made from samples which contained calcium as an impurity. These solutions were titrated with 0.004 N and 0.005 N
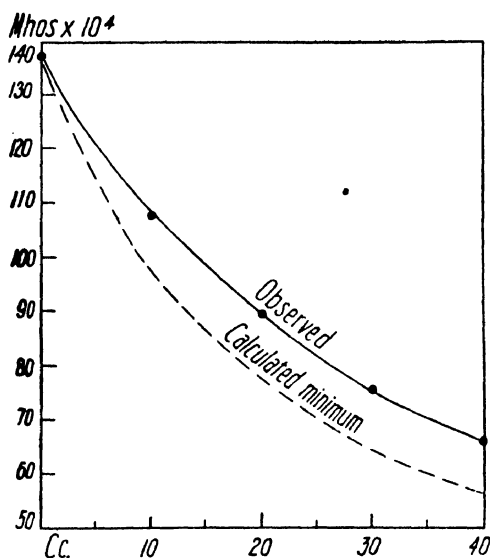
FIG. 1. Titration of untreated serum with 0.01 N CaCl₂
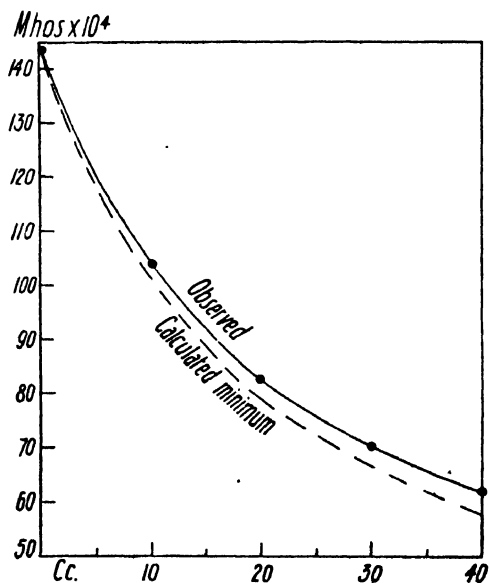
FIG. 2. Titration of serum solution in NaCl with 0.01 N CaCl₂

$CaCl_2$. The curves obtained experimentally were normal. They lay above the calculated minimum curves.

A sample of casein, also containing calcium, was dissolved in a 30 per cent aqueous urea solution. This 5 per cent casein solution was titrated with 0.005 N $CaCl_2$. Here too, the experimental curve was normal; it was higher than the calculated minimum curve.

Serum—Fig. 1 shows the results obtained by titrating untreated serum with 0.01 N $CaCl_2$. This serum contained 9.1 mg. of calcium per 100 cc. and 8.4 per cent protein.
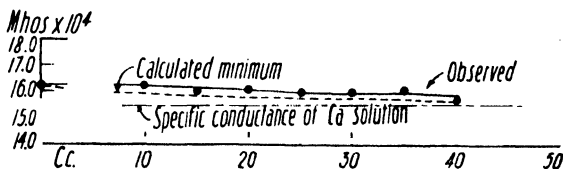
FIG. 3. Titration of serum solution in urea with 0.01 N $CaCl_2$
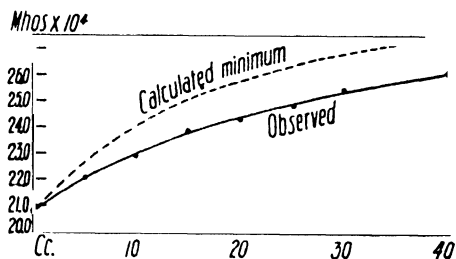
FIG. 4. Titration of serum solution in urea with 0.025 N $CaCl_2$

It is seen that the observed curve is above the calculated minimum curve.

Serum Solutions in NaCl—Fig. 2 is illustrative of the titration curves obtained with serum solutions prepared by dialysis of serum against 3 per cent NaCl for about 5 days followed by dialysis against water for a few hours, and then evaporated to the desired concentration. This particular serum solution contained 0.4 mg. of calcium per 100 cc. The titrating solution was 0.01 N $CaCl_2$.

It is seen that the experimental curve lay above the calculated minimum and that the conductance of the serum solution was quite high, because of its high NaCl content. In subsequent

experiments, therefore, the serum solutions were further dialyzed against 15 per cent aqueous urea solutions to remove the NaCl.

Serum Solutions in Urea—The technique finally adopted gave serum solutions which were almost Ca-free and which contained so little NaCl that the conductance was of the desired range. For example, serum Solution 20 contained 0.5 mg. of Ca per 100 cc.,
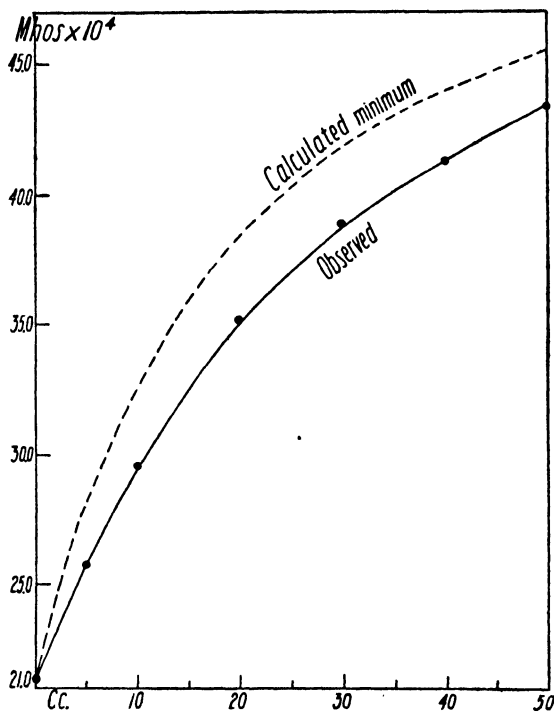
FIG. 5. Titration of serum solution in urea with 0.05 N CaCl$_2$

9.0 per cent of protein, and had a specific conductance of about 20 × 10^{-4} mhos.

Fig. 3 shows the results obtained by titrating this serum solution with 0.01 N CaCl$_2$. The experimental curve was quite close to the calculated minimum.

Another aliquot of this serum solution was titrated with 0.025 N CaCl$_2$; the results are shown in Fig. 4. It is seen that the experimentally obtained values were *below* the calculated minimum

values.   These results are even more strikingly brought out in
Fig. 5 which summarizes an experiment in which another aliquot
of this same serum solution was titrated with 0.05 N CaCl₂.

When such urea solutions of serum are titrated with aqueous
CaCl₂, the urea concentration is diminished, and precipitation of
the proteins may follow.   Furthermore, the diminution of the
urea content in the course of the titration may affect some of the
properties of the solvent.   To avoid these possibilities the CaCl₂
solutions in the foregoing titrations were prepared so as to contain
15 per cent urea.
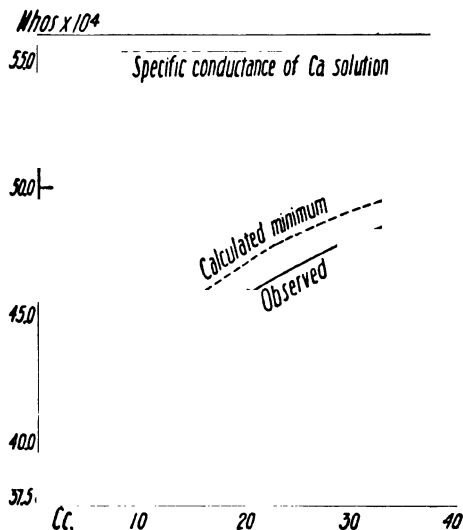
Fɪɢ. 6. Titration of serum solution in urea with 0.05 N CaCl₂

Another serum solution, prepared from a different serum,
contained 5.7 per cent protein and 0.7 mg. of calcium per 100 cc.
When titrated with 0.05 N CaCl₂ it gave results similar to those
described above; *i.e.*, the experimental curve was below the cal-
culated minimum.   The data are summarized graphically in
Fig. 6.   As in the previous titrations, the CaCl₂ solution contained
urea to maintain the constancy of the properties of the solvent.

DISCUSSION

The literature up to the year 1917 on the formation of unionized
calcium-protein complexes was reviewed by Robertson (14) who

discussed the data obtained by the method of electrical conductivity. Since proteins form poorly ionized complexes with acids or bases wrote Robertson[3] then "on adding proteins to a solution of one of these latter, the total number of ions per liter of the solution is diminished . . . the conductivity of the solution is diminished and this diminution affords a measure of . . . the quantity of acid or base bound by the protein." Pauli and Schön (15) employed conductivity measurements in a study of complex formation in the case of $ZnCl_2$ and serum-albumin. They found that "Die Leitfähigkeit der Kombination Salz und Eiweiss liegt ausnahmslos unterhalb der des reinen Salzes. Es verschwinden also dabei Ionen. . ."

The conductivity titrations reported in this paper illustrate the same principle. If the calcium ion concentration is diminished because of the presence of serum, then a reduction in the conductivity should be noted. Since the experimentally obtained curves are lower than the calculated minimum curves (see Figs. 4 to 6), the results obtained are in agreement with the generally held opinion that some of the serum calcium is bound in an unionized complex.

This question of the ionized and bound calcium in serum has been studied by a variety of experimental techniques. In the method of Northrop and Kunitz (16) for determining the amount of calcium bound to protein, a Donnan equilibrium is set up and the membrane potential is measured. They demonstrated that gelatin binds calcium, but so far as we are aware this method has not as yet been applied to the problem of the binding of calcium by serum proteins.

The methods most widely used have been those of dialysis and of ultrafiltration. Among the more recent contributions are the work of Loeb and Nichols (8, 11) and of Morgulis and Perley (17) on dialysis, and of Greenberg and Gunther (18) on ultrafiltration. The latter authors calculated the distribution of protein-bound calcium and ionic calcium in serum from the data of Loeb and Nichols, and concluded that the maximum amount of calcium that serum protein can bind cannot be much greater than the amount of non-diffusible calcium usually present. Robertson,

[3] Reference (14), p. 74.

employing the Ostwald dilution law, found[4] from conductance measurements that sodium serum-globulinate and calcium serum-globulinate have the same degree of dissociation over a wide range of concentrations. On the other hand, Greenberg (19) studied the electrical transference of electrodialyzed serum containing mixtures of sodium and calcium hydroxides and concluded that his results indicated that complex Ca-protein ions exist in such serum solutions.

Solubility studies also furnish evidence in support of the theory that calcium is bound by serum in some unionized form. In 1909, Pauli and Samec (20) treated various slightly soluble calcium salts with water and with serum. They found that the solubility in serum was much greater than in water. Recently, it was found (6) that $CaHPO_4$ is considerably more soluble in serum than in corresponding inorganic serum solutions.[5]

When inorganic serum solutions are shaken with $CaHPO_4$, equilibrium is rapidly reached (5). When serum is similarly shaken with $CaHPO_4$ equilibrium is also rapidly attained, but it is found that $[Ca] \times [HPO_4^-]$ in serum is considerably greater than in corresponding inorganic serum solutions. According to the following considerations this indicates that a large part of the serum calcium is present in unionized form.

In solutions in equilibrium with crystalline $CaHPO_4$, the products of the ion activities must be equal; $i.e.$,

$$(\alpha_{Ca^{++}} \times \alpha_{HPO_4^=})_{serum} = (\alpha_{Ca^{++}} \times \alpha_{HPO_4^=})_{solutions} = K_{s.p.} \, CaHPO_4$$

Since the inorganic serum solutions and sera have the same ionic strength, the activity coefficient $\gamma_{Ca^{++}}$ should have the same value in both systems. The same holds for $\gamma_{HPO_4^=}$. Then

$$([Ca^{++}] \times [HPO_4^=])_{serum} = ([Ca^{++}] \times [HPO_4^=])_{solutions} = K'_{s.p.} \, CaHPO_4$$

where

$$[Ca^{++}] = \frac{\alpha_{Ca^{++}}}{\gamma_{Ca^{++}}}$$

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[4] Reference (14), pp. 226 and 227.

[5] These solutions were prepared so as to contain the inorganic constituents of blood serum in the concentrations in which they occur in serum.

and

$$[HPO_4^=] = \frac{\alpha_{HPO_4^=}}{\gamma_{HPO_4^=}}$$

It was found, however, that at equilibrium

$$([Ca] \times [HPO_4^=])_{serum} > ([Ca^{++}] \times [HPO_4^=])_{solutions}$$

where [Ca] is the total calcium content as given by analysis.[6]
It follows that, in serum in equilibrium with $CaHPO_4$,

$$[Ca]_{serum} > [Ca^{++}]_{serum}$$

or

$$[Ca]_{serum} = [Ca^{++}]_{serum} + [CaX]_{serum}$$

where [CaX] is the concentration of bound calcium in serum.

The calcium ion concentration, $[Ca^{++}]$, was thus calculated for serum as drawn. Equations 3 and 4 of this earlier paper (6) may be combined into the expression

$$[Ca^{++}]_{serum} = [Ca]_{serum} - \left([CaX]_{equil.} \times \frac{7.4 - 6.0}{pH_{equil.} - 6.0}\right)$$

where

$[Ca^{++}]_{serum} =$ concentration of ionized Ca in serum as drawn

$[Ca]_{serum} =$ "   " total   "   "   "   "   "

$[CaX]_{equil.} =$ "   " bound   "   "   "   in equilibrium with crystalline $CaHPO_4$

The results obtained for $[Ca^{++}]$ by this method from the $CaHPO_4$ equilibration experiments are in general agreement with those obtained by other indirect methods, and indicate that a considerable amount of the serum calcium is bound in some sort of complex.

Previous studies showed that the observed specific conductance

[6] In the inorganic serum solutions $[Ca] = [Ca^{++}]$ according to the theory of complete dissociation of strong electrolytes.

was about 2×10^{-4} mhos less than the calculated minimum at a calcium concentration of 0.0025 N in the conductivity titration of $CaCl_2$ and sodium citrate.[7] At this concentration of calcium citrate, about 75 per cent is in the unionized form, according to Wiley. In the conductivity titrations of serum solutions with $CaCl_2$, the observed curve is also about 2×10^{-4} mhos below the calculated minimum. This is about as much of a lowering in the conductance as was obtained in the formation of the Ca-citrate complex. In their study of the Zn-protein complexes, Pauli and Schön (15) used $ZnCl_2$ solutions whose concentrations varied from 4.8 to 58.1 $\times 10^{-3}$ N; the $CaCl_2$ solutions used in the experiments reported in this paper varied from 4.0 to 50.0 $\times 10^{-3}$ N. When such $ZnCl_2$ solutions were mixed with serum-albumin solutions, Pauli and Schön noted reductions in specific conductance which varied from about 5.3×10^{-5} to about 2.6×10^{-4} mhos. The reductions which were noted in the Ca-serum solution conductivity titrations varied from about 1.3 $\times$ 10^{-4} to about 3.0 $\times$ 10^{-4} mhos. The results obtained for the Ca-serum complexes are thus quite similar to those obtained by Pauli and Schön for the Zn-serum-albumin complex.

The question arose as to whether a decrease of 1×10^{-4} mhos in water containing 15 gm. of urea per 100 cc. would indicate the removal of about the same number of ions as the same decrease in specific conductance of an analogous solution which did not contain urea. It was found that the presence of the urea did not markedly affect the specific conductance due to $CaCl_2$. For example, 0.01 N $CaCl_2$ solutions were made up by dilution of the same stock $CaCl_2$ solution. Some of these dilute solutions contained 15 gm. of urea per 100 cc., others contained none. The specific conductance $\times 10^4$ of the different $CaCl_2$ solutions which contained urea were 15.3, 15.5, and 15.6 mhos, while those which contained no urea gave values of 15.9, 15.1, and 15.4 mhos. Thus, within the experimental variations,[8] no effect was noted due to the presence of the urea.

When serum binds calcium ions, which constituent of the serum forms the Ca complex? The consensus of opinion is that the

[7] Reference (3), Fig. 4.

[8] See discussion of the variation in specific conductance of similar $CaCl_2$ solutions on pp. 732 and 733 of reference (3).

proteins combine with the calcium ions to form a Ca-protein complex ion. On the other hand Cameron and Moorhouse (21) suggested that the non-diffusible calcium might be bound to the parathyroid hormone. Morgulis and Perley concluded that there is apparently no basis of fact to such a hypothesis.

Loeb and Nichols (8) performed dialysis experiments in which whole serum, Ca-free serum, and ether-extracted serum were compared as to their capacity to bind calcium "in an endeavor to add evidence in support of the idea that the discrepancy between observed and calculated Ca concentrations in sera is dependent upon the protein fraction of the serum and not upon the lipoids or some other substance (of the type of citrate) combined with calcium." They concluded that the unionized calcium of serum is in the form of Ca-protein complexes, " and that if there are any non-protein factors present their influence must be quantitatively very small."

The binding of calcium ions by serum thus appears to be due to the formation of Ca-protein complexes. It is interesting to note that when the protein and serum solutions were titrated with very dilute Ca solutions, no marked discrepancies in the specific conductances of the mixtures were observed. However, when 0.025 N $CaCl_2$ and 0.05 N $CaCl_2$ solutions were used in the titrations, evidence of complex formation was obtained.

Miss Marion Maurice gave technical assistance in this investigation. Miss Dora Z. Luntz and Mr. Jac Siegel did the nitrogen and calcium analyses.

SUMMARY

1. Serum was dialyzed against 3 per cent aqueous NaCl solution to remove most of the serum calcium and then against 15 per cent aqueous urea solution to remove most of the NaCl.

2. The serum solutions so obtained were almost Ca-free and had a low specific conductance. By evaporation through collodion bags the protein content was increased to any desired extent up to the point of solidification.

3. Conductivity titrations of such serum solutions with 0.025 N and 0.05 N $CaCl_2$ gave values below the calculated minimum values, thus furnishing additional evidence for the binding of calcium ions by serum.

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MANOMETRIC DETERMINATION OF HEMOGLOBIN
BY THE OXYGEN CAPACITY METHOD

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Since Van Slyke (1) originally described a gasometric method for the determination of hemoglobin by analysis of the oxygen-combining power of blood, it has been the practice to saturate the blood with air at atmospheric pressure in a separate vessel and to transfer a sample of the aerated blood to the gas analyzing apparatus.

A method is described here·which requires less time, material, and apparatus, because both saturation with air and the subsequent oxygen analysis are performed in the chamber of the Van Slyke-Neill apparatus (2). Lundsgaard and Möller (3) described a technique of this kind for use with the Van Slyke constant pressure apparatus, but it cannot be used with the Van Slyke-Neill chamber. Saturation by shaking the undiluted blood in the chamber of the latter apparatus filled with air has been found to be impractical; the viscosity leads to emulsion formation with the mercury and foam formation. If the blood is laked and diluted with water, the affinity of hemoglobin for oxygen is so much decreased that saturation with air fails to make the hemoglobin take up its maximum volume of oxygen.

If the blood is diluted with isotonic sodium chloride solution, however, the hemoglobin maintains its normal affinity for oxygen, and can be completely oxygenated by aeration at ordinary pressures. Furthermore, since no frothing or formation of minute air bubbles within the liquid occurs, the material can be agitated without the use of octyl alcohol, and hemolysis is avoided. Blood may be aerated as well as analyzed in the chamber of the Van Slyke-Neill apparatus, in samples of 1.0 to 0.2 cc. The correction for physically dissolved oxygen is large, but it can be so accurately

determined or calculated that its use introduces no significant error. The time required for the complete procedure of saturation and analysis can be reduced to within 2 minutes of that formerly required for analysis alone.

Reagents

0.9 per cent sodium chloride solution.

Ferricyanide Reagent—23.0 gm. of $K_3Fe(CN)_6$ and 8.0 gm. of saponin per 100 cc. of solution.

1 N sodium hydroxide solution.

Sodium Hydrosulfite Solution—This is prepared fresh daily according to Van Slyke ((4) p. 124) and extracted air-free before use.

In the experimental portion of this paper it will be shown that the air-free absorbents for CO_2 and O_2 need not be made fresh daily. If preserved with complete exclusion of air, the NaOH solution may be used for an indefinite length of time. The hydrosulfite solution may be kept for about 15 days.

Procedure for Analysis of 1 Cc. Blood Samples

Aeration of Blood—With the extraction chamber well drained of water and completely filled with mercury, 2.5 cc. of 0.9 per cent NaCl are placed in the cup of the apparatus. Of this, 0.5 cc. is allowed to enter the chamber. 1 cc. of the blood sample is then delivered into the chamber of the apparatus from an accurately calibrated, rubber tipped, stop-cock pipette ((2) p. 532). The slight residue of blood in and near the capillary leading to the chamber is completely washed into the chamber with the rest of the salt solution. The chamber now contains 1 cc. of blood and 2.5 cc. of salt solution. A few drops of mercury are run through the stop-cock of the chamber to dislodge any adherent cells within the bore of, or around the stop-cock. With the stop-cock of the chamber *open to the air* the mercury level is lowered to that point where the constriction of the lower part of the extraction chamber begins, a cm. or so above the 50 cc. mark.

The blood + salt solution mixture is shaken with air at atmospheric pressure for 3 minutes, *at the rate of 350 oscillations per minute.* The mixture is then allowed to run up to within a few

cm. of the stop-cock of the chamber. In order to break up any small bubbles of air which may be present at the surface, 1 drop of octyl alcohol is added to, and gently mixed by hand with the contents of the chamber. The gases are ejected in the manner described by Van Slyke ((5) p. 240) and the stop-cock of the chamber is closed. The blood solution surface is left in the capillary above the stop-cock of the chamber. It is covered with a drop or two of water, then with 1 or 2 cc. of mercury gently delivered from a medicine dropper. A few drops of mercury are run through the stop-cock, which is then closed.

Determination of Oxygen in Aerated Blood + Salt Solution—As described by Van Slyke and Hiller (6), a micro burette is used to deliver 0.13 cc. of the ferricyanide reagent to the blood mixture in the extraction chamber. Since the ferricyanide solution contains no acid, the level of the liquid within the chamber need not be lowered before adding the reagent. Mercury is used to wash in the last portion of the reagent and to seal the stop-cock. The chamber is evacuated, and the O_2 with some CO_2 is liberated by 3 minutes shaking as described by Van Slyke and Neill. The CO_2 is absorbed with 0.5 cc. of air-free 1 N NaOH ((2) p. 555) and the reading p_1 is taken. 1.0 cc. of air-free hydrosulfite is used to absorb the oxygen (Van Slyke (4) p. 124), and the reading p_2 is taken. Both readings are made at the 0.5 cc. mark.

Calculation

Volume per cent combined O_2 in blood $= (p_1 - p_2 - c) \times$ *factor*.

p_1 is the manometer reading after the addition of NaOH in the blood analysis.

p_2 is the manometer reading after the addition of hydrosulfite in the blood analysis.

The factor is taken from Table I, for "Sample = 1 cc., S = 3.5 cc."

c is the $p_1 - p_2$ value obtained by readings before and after the addition of hydrosulfite in a blank analysis. The c correction includes the following four components: (1) the O_2 dissolved by the 2.5 cc. of salt solution, (2) the O_2 in physical solution in the 1 cc. of blood, (3) a trace of O_2 added in solution in the 0.13 cc. of ferricyanide-saponin solution, (4) the fall (of about 1 mm.) of the

mercury level in the chamber caused by adding 1 cc. volume of fluid with the hydrosulfite (the correction for this effect is described on pages 537 and 538 of Van Slyke and Neill (2)).

The *c* correction can be determined by blank analysis in which 0.85 cc. of 0.9 per cent salt solution replaces the 1 cc. of blood. The blank analysis is done with 3.35 cc. of 0.9 per cent NaCl solution in place of 2.50 cc. of salt solution + 1 cc. of blood. The assumption made by Van Slyke and Neill (2) is followed, that the 0.85 cc. of saline solution when aerated takes up in physical solution the same volume of O₂ as 1 cc. of blood. This assumption, if not absolutely exact, introduces no significant error, for oxygen capacities calculated on this basis by both Van Slyke and Hiller ((6) Tables II and IV) and the writer (Table III) have agreed with Van Slyke-Hiller carbon monoxide capacities, which are independent of gas solubility corrections. The physically dissolved O₂ in the saline blood mixture constitutes the greater part of the *c* correction and varies significantly with temperature and barometric pressure, which influence the amount of oxygen dissolved when the saline blood mixture is aerated. Hence, if an empirically determined *c* correction is used, it must be redetermined with each set of analyses.

c Correction Calculated by Nomogram—However, it is not necessary thus to redetermine the *c* correction. The physically dissolved O₂ in the aerated saline blood mixture (sum of components (1) and (2) enumerated above) is the only part of the correction that varies measurably, and it can be accurately estimated from the solubility of oxygen in 0.9 per cent NaCl solution at the observed temperature and barometric pressure. The pressures exerted at 0.5 cc. by the dissolved O₂ extracted from 3.35 cc. of air-saturated 0.9 per cent salt solution are indicated by the nomogram in Fig. 2. A straight line drawn across the temperature and barometer scales cuts the central scale at a point indicating the pressure.

To the pressure correction for dissolved oxygen thus obtained one adds a small constant correction for components (3) and (4) enumerated above, to obtain the total *c* correction. The correction for components (3) and (4) is obtained by a blank analysis in which 3.5 cc. of saline solution, instead of being shaken with air, are first extracted in the *evacuated* chamber to remove dissolved oxygen. The extracted air is ejected from the chamber. The

0.13 cc. of ferricyanide is then added and the analysis is carried through as described above. The fall in pressure caused by addition of the hydrosulfite is added to the correction for dissolved oxygen taken from Fig. 2 to obtain the total c correction. For a given apparatus the fall in pressure caused by addition of hydrosulfite is constant. Consequently, the blank analysis described in this paragraph need be done only once.

The c correction is thus obtained by adding a (= components (1) and (2)) from the nomogram to b (= components (3) and (4)), the constant obtained by the above blank analysis: $c = a + b$. The value of b is about 2.5 mm., when measured with 0.5 cc. of gas space in the chamber.

Construction of Nomogram—The dissolved oxygen in 2.5 cc. of 0.9 per cent NaCl in equilibrium with moist air at a temperature of $t°$ and at the prevailing atmospheric pressure of B mm. is calculated by the equation

$$(1) \quad \text{Cc. } O_2 \text{ dissolved in salt solution} = \frac{20.92}{100} \times \frac{B - W}{760} \times \alpha \times 2.5$$

where W is the vapor tension of water and α is the Bunsen solubility coefficient indicating the cc. of O_2, reduced to $0°$, 760 mm., dissolved by 1 cc. of 0.9 per cent NaCl solution at observed temperature and 1 atmosphere of O_2 pressure.

Similarly, the dissolved oxygen in 1 cc. of blood will be given by the equation

$$(2) \quad \text{Cc. } O_2 \text{ dissolved in blood} = \frac{20.92}{100} \times \frac{B - W}{760} \times \alpha \times 0.85$$

The total dissolved oxygen, reduced to $0°$, 760 mm., may be calculated as volumes per cent of the *blood* in the blood-salt solution mixture by the equation

$$(3) \quad \text{Dissolved } O_2 \text{ in volume per cent of blood} = 20.92 \times \frac{B - W}{760} \times \alpha \times 3.35$$

$$= 0.0922 \, \alpha \, (B - W)$$

This dissolved O_2 when extracted in the gas apparatus and brought to 0.5 cc. volume exerts a pressure P which can be calculated as

$$(4) \quad \frac{0.0922 \, \alpha \, (B - W)}{\text{Factor}} = P$$

With the use, in this formula, of the factors of Table I, Column 4, pressures P were calculated for temperatures from 10° to 35° at

TABLE I

Factors for Calculation of Volumes Per Cent of Oxygen from Pressures in 50 Cc. Apparatus

$a = 0.5$ cc. $i = 1.00$

Temperature	Sample = 0.2 cc. $S = 2.0$ cc.	Sample = 0.5 cc. $S = 2.0$ cc.	Sample = 1.0 cc. $S = 3.5$ cc.
°C.			
10	0.317	0.1269	0.0634
11	16	65	31
12	15	60	29
13	14	56	27
14	13	51	25
15	0.312	0.1246	0.0623
16	10	42	21
17	09	37	19
18	08	33	17
19	07	29	15
20	0.306	0.1224	0.0613
21	05	20	10
22	04	16	08
23	03	11	06
24	02	07	04
25	0.301	0.1203	0.0602
26	00	0.1199	00
27	0.299	95	0.0598
28	98	91	96
29	97	87	94
30	0.296	0.1183	0.0592
31	95	79	90
32	94	75	88
33	93	71	86
34	92	67	83

barometric pressures of 740 and 780 mm. The calculated results are given in Table II. The values of α were calculated from the

data in Landolt-Börnstein-Roth ((7) p. 597). From Table II, Fig. 1 was constructed.

For the construction of Fig. 2, the temperature and barometer scales were laid off. For each assumed unit of P, t at 740 mm. and t at 780 mm. were read on the curves of Fig. 1. Then in Fig. 2, these values of t were connected by a straight line with their corresponding barometric pressures. The intersection of two such lines formed a point on the center line corresponding to a definite unit of P. The calculation was carried out for each whole

TABLE II

Calculation of Pressures Corresponding to Total Dissolved Oxygen Obtained Analytically at Different Temperatures and Atmospheric Pressures

Volume per cent total dissolved $O_2 = (B - W) \times \alpha \times 0.0922$

t	B	Dissolved O_2	Factor	P
°C.	mm.	vol. per cent		mm.
10	740	2.495	0.0634	39.3
15		2.216	0.0623	35.6
20		1.990	0.0613	32.5
25		1.794	0.0602	29.8
30		1.627	0.0592	27.5
35		1.489	0.0581	25.6
10	780	2.632	0.0634	41.5
15		2.338	0.0623	37.5
20		2.100	0.0613	34.3
25		1.894	0.0602	31.5
30		1.719	0.0592	29.1
35		1.574	0.0581	27.1

unit of P, the points were connected to form the central line, and the units of P were then properly subdivided.

Procedure for Analysis of 0.5 Cc. Blood Samples

The technique for the use of smaller blood samples is but slightly different from that described above for 1 cc. samples. The extraction chamber is completely filled with mercury and well drained of water. The sample is carefully delivered into the empty cup of the apparatus from a pear-shaped Ostwald pipette calibrated between marks to deliver 0.5 ± 0.0005 cc. The tip

of the pipette should touch the wall of the cup at a point about 2 or 3 mm. above the level of the blood sample as it is delivered into the cup. The blood is admitted into the chamber. 1.5 cc.
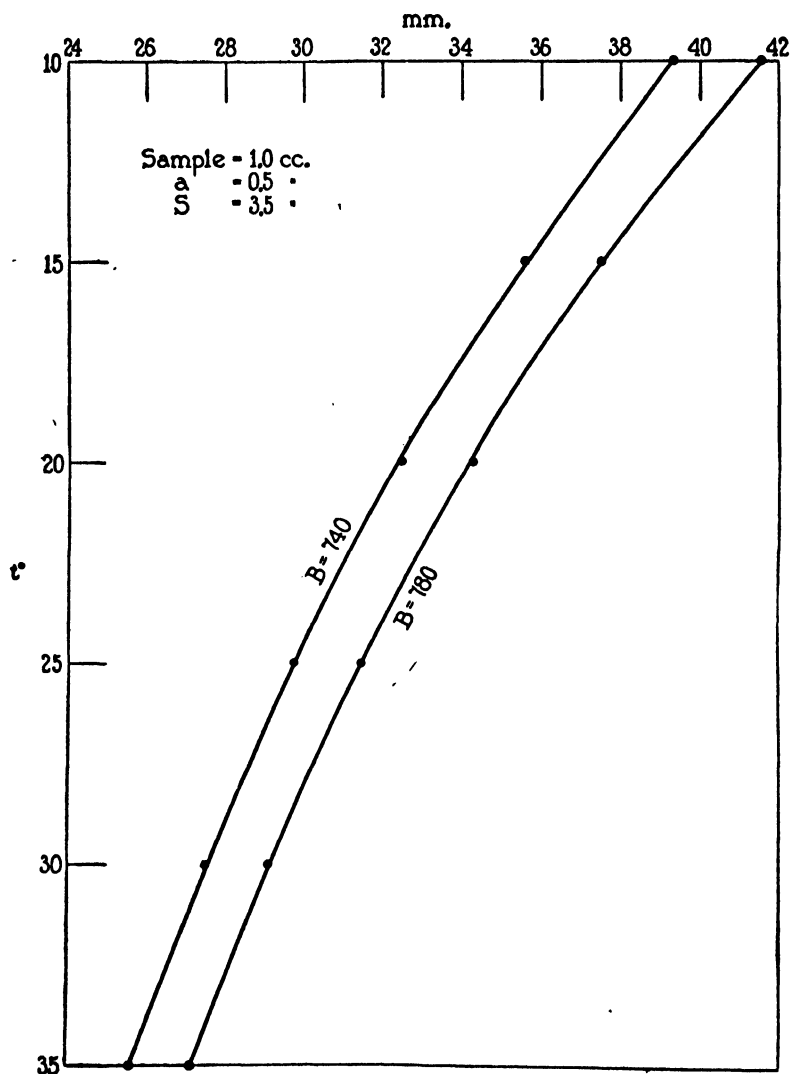
Fig. 1. Curves for dissolved oxygen pressure variation with temperature and barometric pressure; taken from Table II.

of 0.9 per cent NaCl solution added in several portions are then used to wash down into the chamber the cells that have remained in the cup. If necessary, a wire or thin rod may be employed to
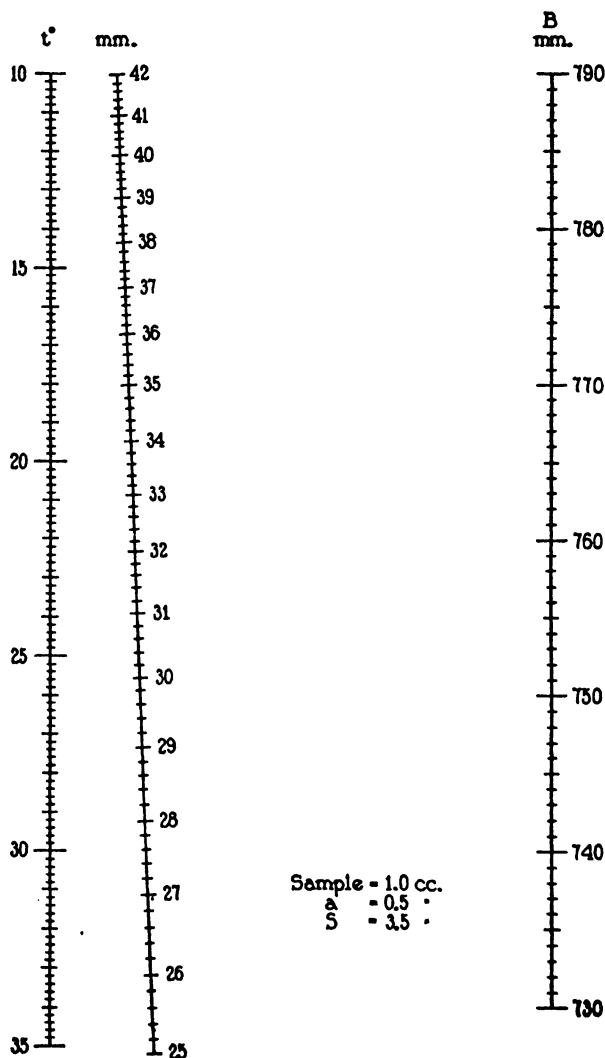
FIG. 2. Nomogram for dissolved oxygen pressures. Sample = 1 cc., a = 0.5 cc., and S = 3.5 cc.

dislodge any adherent cells and to bring them into suspension in
the salt solution.   The procedure from this point on is exactly
the same as that outlined for 1 cc. samples, except that 2½ minutes
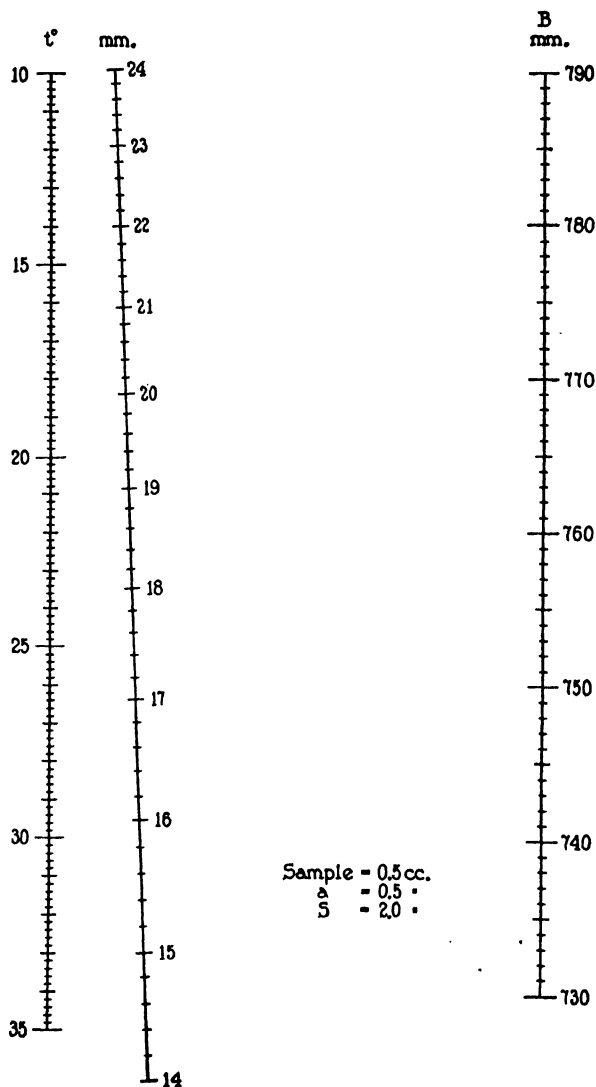
FIG. 3. Nomogram for dissolved oxygen pressures.   Sample = 0.5 cc.,
a = 0.5 cc., and S = 2.0 cc.

suffice for complete saturation of the saline blood mixture with atmospheric air, and only 0.08 cc. of the ferricyanide reagent is necessary to liberate the combined oxygen. Readings p_1 and p_2 are taken at the 0.5 cc. mark.
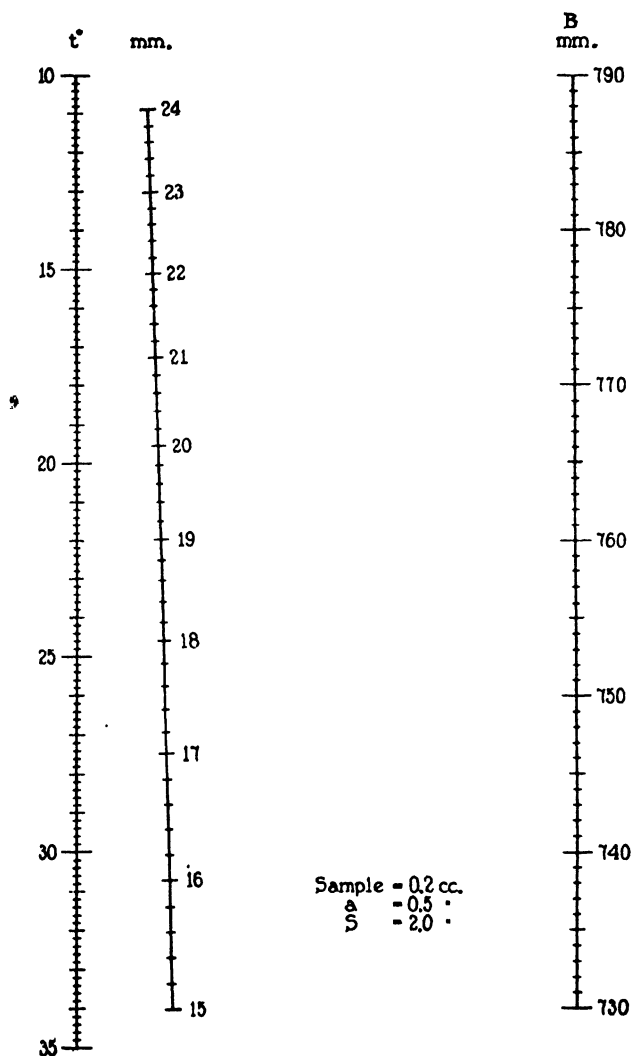
Fig. 4. Nomogram for dissolved oxygen pressures. Sample = 0.2 cc., $a = 0.5$ cc., and $S = 2.0$ cc.

TABLE III

Determination of Hemoglobin in Whole Blood in Terms of Combined O_2 or CO

Sample No.	By method of		
	Author Sample = 1 cc. O_2 capacity	Van Slyke and Neill Sample = 1 cc. O_2 capacity	Van Slyke and Hiller Sample = 2 cc. CO capacity
	vol. per cent	vol. per cent	vol. per cent
1	22.64 22.59	22.48 22.54	
2	21.47 21.53 21.52	21.59 21.54	
3	22.22 22.16 22.14	22.12 22.14	
4	20.99 21.04		20.84 20.87
5	21.60 21.54 21.58 21.53	21.56 21.62	
6	20.64 20.72		20.46 20.59
7	21.33 21.37	21.32	
8	18.46	18.54 18.57	
9	22.22 22.17 22.23		21.93 22.18
10	21.70 21.62 21.63 21.61 21.58 21.55	21.52 21.63 21.58	

TABLE III—*Concluded*

Sample No.	By method of		
	Author Sample = 1 cc. O₂ capacity	Van Slyke and Neill Sample = 1 cc. O₂ capacity	Van Slyke and Hiller Sample = 2 cc. CO capacity
	vol. per cent	*vol. per cent*	*vol. per cent*
11	16.95		
	16.93	17.00	
12	18.69		
	18.66	18.67	
	18.69	18.62	
13	21.67		
	21.54		
	21.50		21.36
	21.65		21.51
14	17.76		17.76
	Sample = 0.5 cc.		Sample = 0.5 cc.
15	13.96		14.06
16	17.85		17.96
17	16.97		
	17.07		16.97

Calculation

Calculation is as described for 1 cc. samples, except that the factors for 0.5 cc. samples in Table I are used, and the *c* correction is determined for the conditions of analysis of the 0.5 cc. sample. The nomogram to be used in this connection is that of Fig. 3.

Procedure for Analysis of 0.2 Cc. Blood Samples

The extraction chamber is completely filled with mercury and well drained of water. 0.8 cc. of 0.9 per cent NaCl solution is placed in the cup. The sample, delivered from a micro pipette calibrated to deliver 0.2 ± 0.0005 cc. between marks, is carefully run underneath the salt solution so that the blood forms a separate layer at the bottom of the cup. The blood, followed by the salt

solution, is then admitted into the chamber. Two portions, of 0.5 cc. of saline solution each, are used to wash adherent cells from the bottom of the cup into the chamber. The procedure from this point on is exactly the same as that outlined for the analysis of 1 cc. samples, except that 2 minutes suffice for complete saturation of the saline-blood mixture with air, and only 0.06 cc. of the ferricyanide reagent need be used. The amounts of alkali and hydrosulfite used as absorbent reagents may be reduced to one-half the volumes used for 1.0 cc. and 0.5 cc. samples.

Calculation

Calculation is as described for 1 cc. samples, except that the factors for 0.2 cc. samples in Table I are used, and the c correction is determined for the conditions of analysis of 0.2 cc. samples. The nomogram to be used in this connection is that of Fig. 4.

EXPERIMENTAL

The order of accuracy and constancy obtained by the present method is indicated by comparison with values given by other methods. In Table III the results for ox and human blood initially reduced with hydrogen, show very good agreement with the oxygen capacity method of Van Slyke and Neill (2), and the carbon monoxide capacity method of Van Slyke and Hiller (6). For the former, O_2 capacity was calculated by subtracting, from the total O_2 values found by analysis, the amount of dissolved oxygen, usually about 0.5 volume per cent at room temperature, as given by the equation

$$(5) \quad \text{Volume per cent physically dissolved } O_2 \text{ in blood} = 20.92 \times \frac{B - W}{760} \times \alpha \times 0.85$$

In Table IV, additional results are given to show that the accuracy of the technique for 0.5 cc. blood samples is about the same as that obtained by the use of 1 cc. samples. The results with 0.2 cc. samples, although less constant, would be satisfactory for most purposes. In these 0.2 cc. analyses of normal blood, the pressure difference due to oxygen, measured at the 0.5 cc. mark, is 65 to 75 mm. A probable error of ± 1 per cent is involved.

Storage of Gas Absorbing Reagents—The necessity of preparing and deaerating fresh alkali and hydrosulfite-absorbent solution

daily can be avoided by keeping the reagents in completely deaerated condition. They can be kept air-free for an indefinite period by storage over mercury. For this purpose, the container suggested by Van Slyke and Neill ((2) p. 535), or the modified Hempel

TABLE IV

Oxygen Capacity Determinations on Small Samples of Blood

Sample No.	Sample = 1 cc. S = 3.5 cc.	Sample = 0.5 cc. S = 2.0 cc.	Sample = 0.2 cc. S = 2.0 cc.
	vol. per cent	*vol. per cent*	*vol. per cent*
1	20.74	20.55	
	20.63	20.63	
	20.77	20.63	
2		21.27	
	21.23	21.11	
	21.19	21.04	
3		21.06	
		21.03	
	20.98	21.07	
	21.00˙	20.97	
4			20.3
	20.18		20.0
	20.20		19.9
5	20.98		20.7
	21.00		20.8
6	21.52		
	21.42		21.6
	21.50		21.8
	21.56		21.5
	21.42		21.6

pipette (6) may be used. The bulbs of the latter should be no larger in volume than 50 cc.

Thus protected, the alkali solution keeps air-free and active indefinitely. However, the hydrosulfite solution eventually decomposes; it cannot be kept in perfect condition for more than 2 or 3 weeks. Table V indicates the results that may be obtained

with oxygen absorbent stored over mercury. Up to 15 days, the solution differed in no respect, in either appearance or ability to absorb oxygen rapidly, from fresh reagent prepared on the day of analysis. After that period, decomposition began to set in, as was indicated by a gradual change in color from deep red to light brown, and the appearance of precipitated material, presumably sulfur. After 20 days from the date of its preparation and deaeration, the material was still able to absorb oxygen quantitatively, but at a slower rate than freshly prepared solution.

TABLE V

Stability of Hydrosulfite + Catalyst Solution for Oxygen Analyses
(a) With freshly prepared reagent; (b) with old reagent.

Experiment No.	Volume per cent O_2	

1	(a) 21.42
	      21.42
		(b) 5 days old 21.52
		                21.50
		                21.56

2	(a) 21.66
	      21.58
	      21.60
		(b) 10 days old 21.48
		                 21.55

3	(a) 21.77
	      21.78
		(b) 20 days old 21.68
		                 21.74

The analytical work in connection with this paper has been done by Mr. Dennis Kertesz.

SUMMARY

A rapid technique for determination of hemoglobin in blood by the oxygen-combining capacity method is described, in which both saturation with air and analysis for oxygen are carried out in the Van Slyke-Neill apparatus. This procedure is made possible by diluting the blood with 0.9 per cent sodium chloride solution before aeration. Compared with the former technique (2) in which the blood was aerated in a separate vessel, the present method requires less apparatus, time, and blood. Good results are obtainable with blood samples as small as 0.2 cc.

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STOICHIOMETRICAL RELATIONS IN THE REACTIONS BETWEEN DYE, NUCLEIC ACID, AND GELATIN

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The nature of the reaction between dyes and proteins has been investigated by Schmidt and his coworkers (1, 2) as well as by Stearn (3–6). The general conclusion is that the union takes place in stoichiometric proportions. The present paper gives the results of a simple procedure, applicable under certain conditions, which demonstrates the validity of the above conclusions in a striking manner.

Stearn (3) and Rehner and Stearn (5) have shown that proteins and dyes undergo displacement reactions of the nature of basic exchange. Thus, for example, when an unionized protein acid is treated with a dye cation, H ion is liberated. The reactions represented by the following equations can be very easily demonstrated by an electrometric measurement.

$$
\begin{aligned}
&(1)\ \mathrm{D^+} &&+\ \mathrm{HPrOH} &&=\ \mathrm{DPrOH} &&+\ \mathrm{H^+} &&\text{(decrease in pH)}\\
&(2)\ \mathrm{D^-} &&+\ \mathrm{HPrOH} &&=\ \mathrm{HPrD} &&+\ \mathrm{OH^-} &&\text{(increase " ")}\\
&(3)\ \mathrm{DOH} &&+\ \mathrm{PrOH^-} &&=\ \mathrm{DPrOH} &&+\ \mathrm{OH^-} &&\text{(" " ")}\\
&(4)\ \mathrm{HD} &&+\ \mathrm{HPr^+} &&=\ \mathrm{HPrD} &&+\ \mathrm{H^+} &&\text{(decrease " ")}
\end{aligned}
$$

Here HPrOH represents isoelectric protein; PrOH⁻ is the protein anion, *i.e.* in a solution at a pH significantly above the isoelectric point; HPr⁺ is the protein cation, *i.e.* below the isoelectric point; D⁺ is the dye cation; D⁻ the dye anion; DOH is free dye base where the dye is weak; and HD, analogously, free unionized dye acid.

The generality of behavior of this kind suggests quite forcibly a chemical reaction, though it is difficult to find conditions which satisfy one that quantitative interpretation is justified. Under any of these conditions, for example, a conductometric titration could not be easily interpreted in any direct manner.

If, however, one chooses a set of conditions where one is dealing with dye cation and protein anion, or *vice versa*, then an ordinary conductometric titration may yield instructive results. Under such conditions we have, if the reaction is as postulated,

$$D^+ + PrOH^- = DPrOH$$
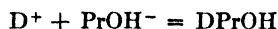
and when this reaction is completed there should result a definite equivalence point.

EXPERIMENTAL

In this experiment Eastman ash-free gelatin (0.08 per cent residue), Eastman yeast nucleic acid (residue free from all but traces of chlorides and sulfates), and National Aniline and Chemical Company methyl violet were used. Solutions of the latter were made up on the basis of nitrogen content.

The measurements were made with a Wheatstone bridge. Curtis wound coils from Leeds and Northrup furnished the standard resistance and telephone receivers the indicating instrument. Temperatures were maintained constant to 0.05°. The solutions were adjusted by means of NaOH to a pH of 7 ± 0.2. At this value small pH changes will not appreciably affect conductivity values and it has been shown (3, 6) that both gelatin and nucleic acid are sufficiently ionized under these conditions so that the change in pH is small on addition of basic dye salt. Titrations were made in both directions; *i.e.*, the gelatin and the nucleic acid were titrated with the dye, and also the dye was titrated with the gelatin and the nucleic acid. The solutions were not allowed to stand after being made up (except for the dye) any longer than necessary for attaining bath temperature before the titrations were made. The stock gelatin solutions were 1 per cent and the nucleic acid solutions 0.5 per cent. Inasmuch as there was an appreciable dilution during the titration the conductivity values were corrected to the original volume. This procedure involves almost the only assumption in the work, but inasmuch as the dilution was such that it did not greatly alter the salt environment it is thought to be justified. The only particular precaution necessary in carrying out a titration is that the electrodes of the cell be kept free from a coating of the dye proteinate or dye nucleate precipitate which forms when the concentrations are high.

In Fig. 1 are plotted data for one of the titrations with nucleic acid.

Table I gives a résumé of the results of this titration and also of one in which solutions only half as concentrated were titrated.
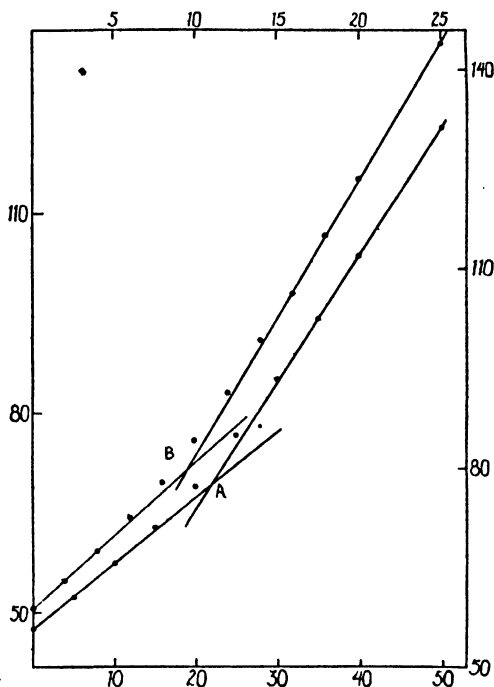
FIG. 1. Curve A, titration of 10 cc. of 0.5 per cent nucleic acid diluted to 300 cc. with 0.007 M methyl violet. Coordinates are on the lower and right-hand scales. The equivalence value is 22.0 cc. Curve B, titration of 20 cc. of 0.007 M methyl violet diluted to 300 cc. with 0.5 per cent nucleic acid. Coordinates are on the upper and left-hand scales. The equivalence value is 9.25 cc. Abscissæ are cc. of titrating solution and ordinates are conductances times 10^6 in reciprocal ohms.

Values in Column 3 of Table I are obtained from the expression $0.007 \times \dfrac{10}{m}$, where values of m are those in Column 2 and 0.007 is the dye concentration; those in Column 4 are obtained from the expression $\dfrac{5}{n}$, where values of n are those in Column 3, and the 5 is the number of gm. of the nucleic acid per liter of stock solution.

TABLE I
Résumé of Titrations of Nucleic Acid with Methyl Violet

(1)	Nucleic acid used per 10 cc. dye (2)	Apparent concentration of nucleic acid (3)	Apparent equivalent weight of nucleic acid (4)
	cc.	eq. per l.	gm.
1. Acid with dye (Fig. 1)..........	4.55	0.0154	325
2. Dye " acid (" 1)..........	4.62	0.01515	330
3. Acid " dye (more dilute solution)........................	4.82	0.01453	344
4. Dye with acid (more dilute solution)........................	4.75	0.01475	339
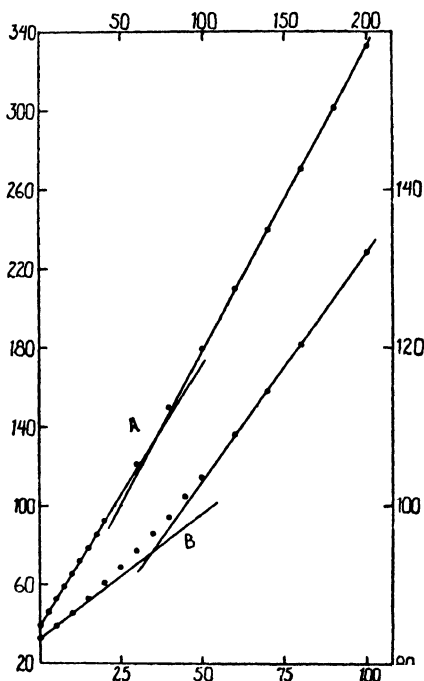
FIG. 2. Curve A, titration of 50 cc. of 1 per cent gelatin solution diluted to 250 cc. with 0.005 M methyl violet. Coordinates are on the upper and left-hand scales. The equivalence value is 71 cc. (The angle here is so large that this point is somewhat difficult to locate precisely. There is no doubt but that it lies within 0.5 cc. of this value.) Curve B, titration of 50 cc. of 0.005 M methyl violet diluted to 250 cc. with 1 per cent gelatin solution. Coordinates are on the lower and right-hand scales. The equivalence value is 35.0 cc. Abscissæ are cc. of titrating solution and ordinates are conductances times 10^6 in reciprocal ohms.

If we take Levene's formula for yeast nucleic acid (7), its molecular weight is 1303, giving, per phosphoric acid group, a weight of 326. The values above when averaged are somewhat higher than this, but there is little way to check the purity of the preparation. The close approach to this value in the case of the more concentrated solutions is probably fortuitous, though, other things being equal, the deviations in Table I for any one concentration should indicate the probable precision of the method. In the more dilute solutions there was little apparent precipitation of the dye nucleate, and perhaps a slightly greater dissociation of the salt accounts for the higher values obtained here.

Fig. 2 represents data from a titration of gelatin. These results give, since the dye was 0.005 M and the gelatin solution contained 10 gm. per liter, the respective values of 71.0×10^{-5} and 71.4×10^{-5} equivalents of dye bound per gm. of gelatin. They are in essential agreement with the value 70.0×10^{-5} reported by Rawlins and Schmidt (2) for a pH of 11.8 though the pH here was only about 7. The results of these workers, however, indicate that the amount of basic dye bound by gelatin is practically independent of pH through quite a wide range provided one is 2 to 3 units removed from the isoelectric point.

DISCUSSION

The method outlined here is independent of the solubility relations of the dye compounds formed. In the one case the titration ends with a large excess of dye, and in the reverse titration there is finally a large excess of the nucleic acid or of the gelatin with its protective action against precipitation, and yet the significant fact of closely checking values should be noted. Indeed, while no quantitative estimation of the relative amounts of precipitation was attempted, it seemed superficially that there were large differences in the amount of precipitate formed in the various titrations, yet the results were in good agreement. This seems to indicate a covalent linkage between dye ion and nucleate or proteinate ion, regardless of solubility relations.

Chapman, Greenberg, and Schmidt (1) assume that the action of acid dyes on proteins is analogous to that of simple acids such as HCl. The present author would like to point out another

possibility which fits the results of these authors as well as their own assumption, and is more in keeping with his own results on ionic displacement mentioned above (3, 5, 6). Such results indicate that dye cation replaces H ion and that dye anion replaces OH ion in proteins. This would indicate that the mechanism of the binding of simple acids corresponds more nearly to that of basic dyes than it does to acid dyes, in both cases the cation being the important reagent ion. With acid dyes and with simple bases such as NaOH it would be the anions of the reagents which are important. This concept would in no way affect correlations of binding power with amino acid content of the proteins. Furthermore, the fact that basic dyes are more completely bound in alkaline solution than in acid solution does not affect the argument since in acid solution there is great mass competition of H ion with the basic dye cation, and in alkaline solution the same is true between OH ion and acid dye anion. This would mean, of course, that there should be no abrupt discontinuities in the binding behavior of proteins at their isoelectric points, but such a consequence is in accord with the findings of Chapman, Greenberg, and Schmidt (1), and Stearn (4) has shown that a sufficient excess of basic dye will completely "neutralize" gelatin at its isoelectric point, and seemingly at pH values distinctly more acidic.

SUMMARY

1. Conductometric titrations of methyl violet with sodium nucleate and sodium gelatinate were made.

2. Definite equivalence values were found which were independent of the direction of the titration.

3. The equivalent weight of nucleic acid from these data is in good agreement with that calculated from Levene's formula.

4. The binding power of gelatin for basic dye found here is in good agreement with that obtained by Chapman, Greenberg, and Schmidt.

5. The conditions under which the technique used here is applicable are discussed.

6. The results indicate combination of dye with nucleic acid or with gelatin in stoichiometric proportions.

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STUDIES OF THE INTERMEDIATE PRODUCTS FORMED DURING THE HYDROLYSIS OF UREA BY UREASE*

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Cyanic Acid Is Not Formed by the Action of Urease on Urea

In 1923, Fearon[1] stated that he had been able to detect traces of cyanic acid in solutions in which urea was being decomposed by soy bean urease. He proposed that the reaction concerned is not a true hydrolysis, but rather a cleavage of the urea molecule into cyanic acid and ammonia and that the cyanic acid is at once spontaneously hydrolyzed to ammonia and carbon dioxide.[2]

Sumner,[3] in 1926, objected to Fearon's theory not only because he could not detect cyanic acid after allowing exceptionally pure preparations of urease to react with urea, but also because he contended that cyanic acid, if formed, would not be hydrolyzed in an alkaline or neutral medium, but would accumulate so that the end-product of urease action would be ammonium cyanate and not ammonium carbonate. Sumner is not the only author whose opinion and results are contrary to those of Fearon. Armstrong and Horton,[4] in 1912, failed to detect cyanic acid among the decomposition products of urea acted on by urease and Iwanoff,[5] in 1924, could find no cyanic acid when urease was allowed to act upon urea dissolved in 80 per cent alcohol. Kay[6] has ob-

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* The work described in this paper has been made possible by a grant from the Heckscher Research Council.

[1] Fearon, W. R., Biochem. J., **17**, 84, 800 (1923).

[2] Werner, E. A., The chemistry of urea, New York (1923).

[3] Sumner, J. B., J. Biol. Chem., **68**, 101 (1926).

[4] Armstrong, H. E., and Horton, E., Proc. Roy. Soc. London, Series B, **85**, 109 (1912).

[5] Iwanoff, N. N., Biochem. Z., **150**, 108 (1924).

[6] Kay, H. D., Biochem. J., **17**, 277 (1923).

jected to Fearon's theory because of the great rapidity and completeness with which urea is hydrolyzed by urease.

In 1926, Fearon stated in another paper[7] that he fully realized the validity of the objections of Kay and of Sumner, but that he had been able to demonstrate the formation of traces of cyanate by the use of a new color test. The test could not be used directly because the soy bean meal added as a source of urease interfered. Fearon allowed the meal to react with urea for 10 hours, after which the solution was treated with silver nitrate. The precipitate was decomposed by adding a sufficient amount of hydrochloric acid and the color test was then applied to the solution.

Fearon worked under the disadvantage of having to employ an impure urease preparation. Using crystalline urease[8] we have had no difficulty in applying Fearon's test in slightly modified form directly to solutions of urea that are undergoing hydrolysis by urease and in showing that within the limit of delicacy of the test no cyanic acid is ever present.

The original test described by Fearon states that one should add 2 to 6 drops of a 6 per cent alcoholic benzidine solution to 5 cc. of water, followed by 2 to 6 drops of cupric acetate and that, after mixing, the solution suspected of containing the cyanate should be added. In the presence of cyanate a purplish precipitate appears. We have found it necessary to use much less copper acetate because if too much is used there is formed in the presence of ammonium chloride a blue, green, or gray precipitate which spoils the test. In our experiments large amounts of ammonium chloride were present.

Our procedure consisted in adding to 2 gm. of urea 5 cc. of water, 3 drops of 0.04 per cent methyl red, and 1 cc. of phosphate-free crystalline urease of from 50 to 150 units activity. After being mixed, 0.1 N hydrochloric acid was run in, drop by drop, with · constant rotation to neutralize the ammonia, care being taken never to make the solution acid to methyl red. We have usually added about 30 cc. of acid. To stop the action of the urease we have next added 1 drop of cupric acetate, after which 0.1 N hydrochloric acid has been added cautiously to bring the solution to pH 5. Cyanate has then been tested for by adding 1 drop of

[7] Fearon, W. R., J. Biol. Chem., 70, 785 (1926).
[8] Sumner, J. B., J. Biol. Chem., 69, 435 (1926).

freshly prepared 6 per cent alcoholic benzidine to 3 cc. of the solution. In all cases the tests, as indicated by lack of a purplish precipitate, have been negative.

As a check of the delicacy of the test runs have been made similar to the above except that known amounts of pure potassium cyanate have been added at the start. It was found that the purple precipitate was obtained if the final solution contained as much as 0.13 mg. of potassium cyanate per cc. Pure potassium cyanate solutions gave positive tests with as little as 0.025 mg. per cc.

We have employed the silver nitrate test for cyanate in a similar manner, but here have neutralized the ammonia with 0.2 N acetic acid and have removed the urease by means of aluminum hydroxide before adding the silver nitrate. In all cases the tests were negative as shown by lack of a precipitate. When potassium cyanate was added at the beginning of the experiment a precipitate of silver cyanate was formed if as much as 0.15 mg. of cyanate was present in the final solution.

Ammonium Carbamate Is Produced by the Action of Urease on Urea

Whereas the hydrolysis of urea by water, acids, and alkalis is best explained by the intermediate formation of ammonium cyanate,[9,10] the intermediate product of the hydrolysis of urea by urease has been considered by the majority of observers in this field to be ammonium carbamate. As early as 1885 Fenton[11] suggested that ammonium carbamate is formed by the hydrolysis of urea under the influence of ferments. Armstrong and Horton[4] stated that ammonium carbamate is the only intermediate compound which should be considered and that urease, by determining the resolution of urea in an abnormal direction, serves a most important purpose..

As far as a search of the literature shows, Yamasaki[12] has been the only investigator who has made quantitative analyses to show that carbamate is actually formed from urea by urease. He used the method of Fenton[11] for the determination of urea and carba-

[9] Fawsitt, C. E., *Z. physik. Chem.*, **41**, 601 (1902).

[10] Burrows, G. J., and Fawsitt, C. E., *J. Chem. Soc.*, **105**, 1, 609 (1914).

[11] Fenton, H. J. H., *Proc. Roy. Soc. London*, **39**, 386 (1885).

[12] Yamasaki, E., *Tohoku Imperial Univ. Sc. Rep.*, series 1, **9**, 96 (1920).

mate. He added carbon dioxide to solutions of urea that were being hydrolyzed by soy bean urease and found this to have a retarding effect upon the spontaneous decomposition of the carbamate. He found that the concentration of the carbamate reached a maximum and that its rate of decomposition was measurable. Yamasaki calls his methods semi-quantitative, nevertheless his findings have been fully confirmed by us.   •

We felt that the experimental work showing ammonium carbamate to be the intermediate product should be repeated, using crystalline urease and, if possible, employing a method more dependable than that used by Yamasaki.

Method for Determination of Carbamic Acid

The first step necessary was to find some reliable way of analyzing for carbamic acid. At the start we used in a qualitative manner the procedure outlined by Lewis and Borrows.[13] Here, carbonate is removed by adding ice-cold barium hydroxide and filtering in the cold. If the clear filtrate is warmed gently, or allowed to stand out of contact with the air, the carbamate decomposes and gives a precipitate of barium carbonate. We intended to adapt this procedure for quantitative use but did not because we were successful in finding a better one. Our method depends upon the fact that when a solution of ammonium carbamate is Nesslerized only the nitrogen of the ammonium radical reacts. The carbamic acid is stabilized by the Nessler reagent and does not decompose for as long as 1 hour.[14]

To carry out the analysis one pipettes 1 cc. of the carbamate solution directly into a 100 cc. volumetric flask which already contains 3 drops of saturated sodium hydroxide mixed with about 70 cc. of water. The alkali serves to stabilize the carbamate. The solution to be analyzed should contain approximately 1 mg. of ammonia nitrogen. The standard should contain the right amount of ammonium sulfate and the same amount of sodium hydroxide, urea, and enzyme as the unknown. The enzyme should be inactivated by potassio-mercuric iodide before being added to

[13] Lewis, G. N., and Borrows, G. H., *J. Am. Chem. Soc.*, **34**, 1515 (1912).
[14] Sumner, J. B., and Hand, D. B., *Proc. Soc. Exp. Biol. and Med.*, **27**, 292 (1930).

the standard.   Both flasks are treated with 10 cc. of the Nessler solution of Folin and Wu, diluted, mixed, and read in the colorimeter.   This reading gives the value for ammonium nitrogen.   To obtain the value for the total of ammonium and carbamate nitrogen one pipettes 1 cc. of the carbamate solution into a 100 cc. volumetric flask which contains 1 cc. of N hydrochloric acid.   The carbamate is instantly decomposed to ammonium chloride and carbon dioxide.   The solution is Nesslerized and compared with a standard containing the same amount of acid and an appropriate amount of ammonium sulfate, urea, etc.   In experiments where the carbamate is increasing or decreasing rapidly the two analyses must be made by two persons at the same time, or else the result of one analysis must be interpolated to the time of the other.   Subtraction of the value of the analysis to which alkali was added from the result of that to which acid was added gives the carbamic acid nitrogen in mg. per cc.

The method has been shown to give accurate results with pure ammonium carbamate.   Here the salt is dumped from a weighing bottle into dilute sodium hydroxide and the solution mixed and analyzed.   The method is not accurate for determining carbamate when the carbamate amounts to as little as 10 per cent of the total of ammonium and carbamate nitrogen, since the determination is by difference.

Our pure ammonium carbamate was prepared by passing ammonia gas, dried over sodium hydroxide sticks, and carbon dioxide, dried by bubbling through strong sulfuric acid, into a bottle. The solid carbamate was loosened from the bottom of the bottle by pounding with a glass rod.   Samples of the salt dissolved in barium hydroxide without giving a precipitate of carbonate.

Proof That Carbamate Is the Intermediate Product

With the procedure described above it has been shown that when urease acts on urea in alkaline solution ammonium carbamate is formed in large quantities and that after the urease has been inactivated the carbamate decomposes to carbonate at a measurable rate.

Experiment 1—100 cc. of 1 per cent urea chilled by crushed ice were treated with 3 cc. of crystalline urease of about 50 units per cc.   The solution was mixed at once.   After 5 minutes the

urease was inactivated with 1 drop of 17 per cent potassio-mer-curic iodide. Samples were taken at intervals and analyzed for carbamate by our procedure. The percentages of ammonium
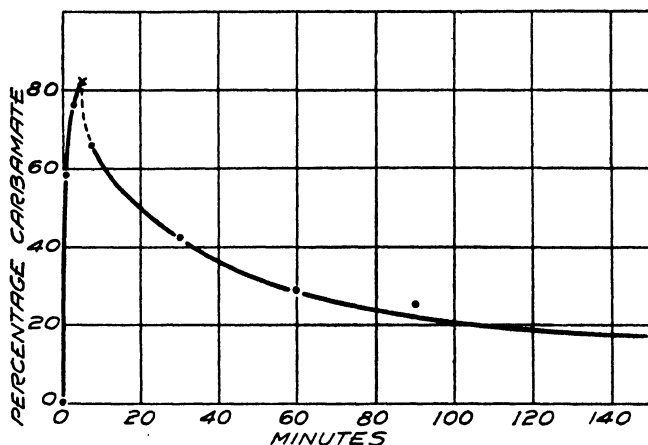
FIG. 1. Formation of carbamate and destruction after poisoning urease

FIG. 2. Formation of ammonium carbamate by urease

carbamate, of the total of ammonium carbonate and carbamate are plotted in Fig. 1.

Experiment 2—In Fig. 2 are shown results of an experiment

similar to the above except that the urease was not poisoned with potassio-mercuric iodide.

Experiment 3—Fig. 3 shows the spontaneous hydrolysis of ammonium carbamate after addition of 261 mg. of the pure salt to 100 cc. of water at 0°.

Experiment 4—We wished to see if any carbamate could be detected when urea is decomposed by urease at 0° in the presence of phosphate buffer or acetate buffer, but no trace of carbamate could be detected either by our method or by the more sensitive method of Lewis and Borrows. Hence it appears that under
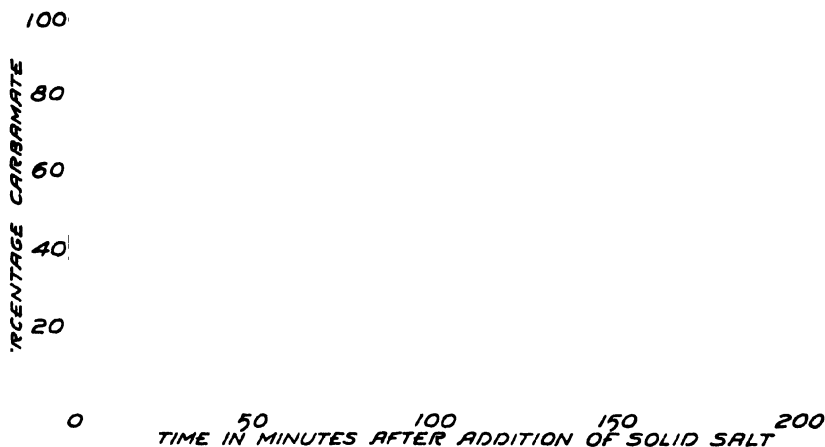
Fig. 3. Decrease in percentage of carbamate with time

these conditions carbamate is not formed, or, what is more probable, that carbamate is decomposed as fast as it is formed.

Experiment 5—We were interested to see if the spontaneous decomposition of carbamate could be prevented by the presence of an alkaline buffer. A buffer solution just alkaline to phenolphthalein was made from pure sodium bicarbonate. This was chilled to 0° and into it were dumped 191 mg. of ammonium carbamate. Fig. 4 shows that the rate of hydrolysis is hastened by the buffer, since after 16 minutes nearly all of the carbamate has become decomposed.

Experiment 6—Yamasaki states that carbonic acid somewhat prevents the spontaneous hydrolysis of carbamate. We tested

this by dumping 220 mg. of ammonium carbamate into 100 cc. of water at 0° through which a rapid stream of carbon dioxide was passing.   After 6 minutes the reaction of the solution became
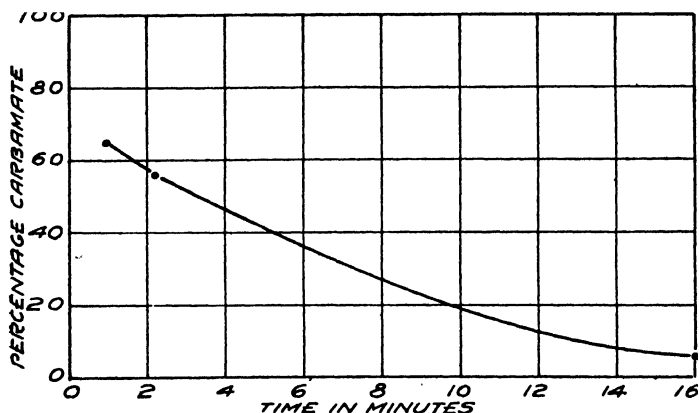
FIG. 4. Decrease in percentage of carbamate in Na_2CO_3-$NaHCO_3$
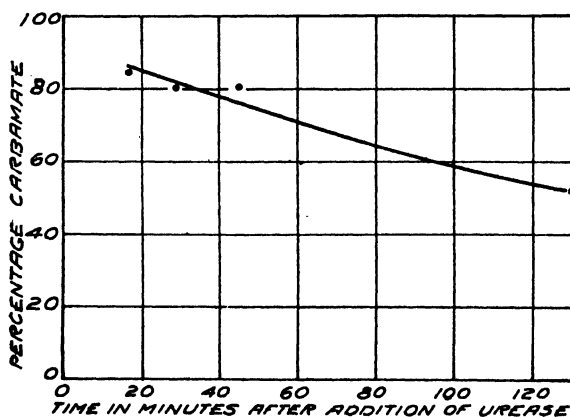
FIG. 5. Decrease in percentage of carbamate in presence of CO_2

acid to phenol red.   Analyses made 7 and 24 minutes after the addition of the carbamate gave respectively 87 and 84 per cent ammonium carbamate, showing that carbonic acid delays the decompositon of carbamic acid.

Experiment 7—Here 100 cc. of 0.2 per cent urea were chilled to 0° and saturated with carbonic acid. 1 cc. of urease was added at the rate of 1 drop per minute. After 15 minutes the urease was inactivated by 1 drop of potassio-mercuric iodide. Analyses were made at intervals. As can be seen from Fig. 5 the rate of hydrolysis of carbamate is decreased in this experiment.

The possibility exists that the ammonium carbamate formed by the action of urease on urea is a secondary product produced from carbon dioxide and ammonia, although it is difficult to understand what primary substance there could be that would split into carbon dioxide and ammonia. In Experiment 8 it is shown that when ammonia is treated in the cold with carbonic acid the primary product is largely ammonium carbamate.

Experiment 8—Ice water was saturated with a stream of carbon dioxide. Ammonium hydroxide was added in small quantities at a time until the total nitrogen of the solution amounted to 1 mg. per cc. The solution, which at this time was acid to phenol red, contained 82 per cent of its nitrogen in the form of ammonium carbamate.

SUMMARY

We have shown that cyanate is not formed during the hydrolysis of urea by urease.

We have confirmed the work of Yamasaki and have shown that when urea is acted on by urease in alkaline solution ammonium carbamate is formed in large amount and that the carbamate spontaneously decomposes at a measurable rate. We have confirmed Yamasaki's finding that carbonic acid delays the decomposition of carbamate. No carbamate is formed by urease in the presence of buffers. There is no reason to doubt that ammonium carbamate is an intermediate product of the action of urease on urea, but it is not necessarily the first intermediate product.

POTENTIOMETRIC STUDIES ON COMPLEX IRON SYSTEMS

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Outlines of Theory

In a solution containing both ferric and ferrous ions the oxidation reduction potential is controlled by that special form of Nernst's equation, which often is called Peters' equation.

$$E = E_0 + \frac{RT}{F} \ln \frac{[Fe^{+++}]}{[Fe^{++}]} \tag{1}$$

Brackets mean activities. Provided the total amount of iron is very small compared with the concentration of an indifferent electrolyte, which is kept constant while the ratio Fe^{+++}: Fe^{++} is varied, concentrations may be taken instead of activities. In a solution of a ferric compound of a given concentration, it is almost impossible to determine the free ferric ions because of the great tendency of complex formation with the various anions. The same difficulty, though to a much smaller extent, prevails with respect to the ferrous ions. In general, the combination of a ferric or ferrous ion with the complex-forming acid is reversible. If the complex is formed by one ferric (or ferrous) ion and a definite number of the complex-forming anions, the following double equation holds.

$$E = E_1 + \frac{RT}{F} \ln \frac{[Fe^{+++}]}{[Fe^{++}]} = E_2 + \frac{RT}{F} \ln \frac{[\text{ferri complex}]}{[\text{ferro complex}]} \tag{2}$$

where $[Fe^{+++}]$ and $[Fe^{++}]$ signify the concentration of the free iron ions. If a complex be formed with a nucleus of 2 or more Fe atoms, or, if several complexes can be formed, this equation may

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correspondingly be modified, but the above type of equations holds anyway and we may restrict ourselves to the simple case represented by equation (2).

Only in a few cases is the complex formation practically complete even without an excess of the complex-forming anion. This is true for CN^- as complex-forming anion, also for the α-α'-dipyridyl complex of iron (1) and for all iron compounds related to hematin. In most cases the affinity of the complex formation is weaker, and even the number of anions attached to the iron atom may vary according to conditions of concentration. In such a case, various complex compounds may exist simultaneously in the solution. In order to simplify the matter all of our experiments were carried out in solutions containing a small concentration of Fe (0.001 gm. atom per liter) and a very high concentration of the complex-forming anion (about 0.1 M) so that the formation of only one complex, namely the highest, was favored.

As long as this condition is fulfilled, the following statement can be maintained. When in such a solution the total amount of Fe and of the complex-forming anions is kept constant but the ratio of $Fe^{II}:Fe^{III}$ is varied, the ratio of free ferric ions to the ferric complex compound is constant, and the ratio of free ferrous ions to the ferrous complex is constant also. These two constants will be, in general, different from each other. In general, ferric ion has a greater affinity for complex formation than ferrous. So,

$$[\text{Ferri complex}] = k_1 \, [Fe^{+++}]$$

$$[\text{Ferro complex}] = k_2 \, [Fe^{++}]$$

If $k_1 = k_2$, the addition of the complex-forming anion to a solution containing Fe^{+++} and Fe^{++} in the free ionic state, would not change the potential. On the other hand, if $K_1 \neq K_2$ (and usually $K_1 \gg K_2$) equation (2) may be written

$$E = E_1 + \frac{RT}{F} \ln \frac{[Fe^{+++}]}{[Fe^{++}]} = E_2 + \frac{RT}{F} \ln \frac{K_1 \, [Fe^{+++}]}{K_2 \, [Fe^{++}]}$$

Hence, $E_1 - E_2 = \dfrac{RT}{F} (\ln K_1 - \ln K_2)$. With log K as a measure of the complex affinity, $E_1 - E_2$ is a measure for the difference of the complex affinity of the ferric, and of the ferrous ion, for the

complex-forming anion concerned. This may be also expressed in this way: the more the complex affinity of the ferric ion for a complex-forming anion exceeds the complex affinity of the ferrous ion, the smaller becomes E and the more negative is the potential range of a ferri-ferro mixture of that complex.

It is understandable that the potential range of a ferri-ferro system may lie anywhere according to the chemical nature of the anion. In fact. ferric iron in the form of the chloride belongs to the strong oxidants, whereas ferrous iron in form of the pyrophosphate, oxalate, or tartrate belongs to the strong reductants. On the other hand the ferrous complex of α,α'-dipyridyl can be oxidized only by the most powerful oxidants such as free chlorine (1).

Though such a statement is not at all novel,[1] the systematic knowledge of various ferric-ferrous systems is rather restricted. A great deal of work has been devoted to the system ferric-ferrous chloride and to ferri-ferrocyanide. Recently various penta-cyanide complexes have been studied by Davidson (2). A preliminary study for iron pyrophosphate has been carried out by Spoehr and Smith (3) in which the authors encountered certain difficulties, especially in measuring pH of the solutions. Earlier potentiometric studies were made on fluoride by Peters (4). Conant's (5) research on the potential of hemoglobin-like compounds may be included in this field. He encountered great difficulty in getting steady potentials. It was in the hope to prepare the field for successful continuation of those important studies, inaugurated by Conant, that we undertook the following studies on simple complex iron compounds.

Influence of pH upon Potential

The complex itself, as a rule, is an acid or a base. In all cases investigated in the experimental part of this paper, it is always an acid, and even a bivalent or polyvalent one, and therefore is capable of existence in various states of electrolytic dissociation. If this is the case, in equation (2) [ferri complex] signifies only one (though any one) of the possible states of electrolytic dissociation, and [ferro complex] then signifies that form of the reduced complex, which differs from the latter only by an electron and by

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[1] Reference may be made particularly to Foerster, F., Elektrochemie wässriger Lösungen, Leipsic, 4th edition, 214 (1923).

nothing else. As the dissociation constants of the complex anion will be, in general, different for the ferric and the ferrous state, the potential of the system must depend on pH in the same way as Clark and Cohen (6) have shown to be the case for reversible dyestuff systems.

Besides, however, in our systems, there is another influence of pH noticeable. Ferrous, and even more, ferric ion has a strong affinity for OH^- ions with which it forms the practically insoluble hydroxide. There is, therefore, always a competition for the iron, of the hydroxyl ion and the complex-forming ion in question. For this reason, only very tight complexes are stable in a somewhat alkaline solution (oxalate, pyrophosphate), whereas others can exist only in acid solutions (salicylate). On the other hand, there is a competition for the complex-forming anion of the hydrogen ions and of iron ions. This sets a limit to an iron complex formation with increasing acidity, also. So in the case of a rather weak complex former (such as salicylic acid) there is only a certain range of pH in which the complex formation takes place to an appreciable extent. This complicated situation renders it impossible to interpret the influence of pH upon the potential in a generally satisfactory way. The interpretation of the sometimes very complicated shape of the E_0-pH curve for reversible dyestuffs according to Clark is based on the assumption that the whole may be thought of as made up by segments of straight lines the slopes of which differ by 0.03 volt per pH unit. The theory underlying this assumption is no longer valid in the present case, in which variation of pH not only influences the electrolytic dissociation of the complex acid but also influences the (non-electrolytic) dissociation of the complex molecules into their constituents.

In contrast with the complexity of the E_0-pH curves, each single titration curve at constant pH is quite regular. To speak more strictly, the following statement can be made. When the ferric complex (formed in the solution in presence of a large excess of the complex-forming anion) is titrated with a reductant at constant pH and the ionic strength of the solution is approximately constant during the titration, the potential depends on the amount of added equivalents of the reductant, x, in this way,

$$E = E_0 + \frac{RT}{F} \ln \frac{a - x}{x}$$

throughout the whole range of titration independent of the individuality of the electrode, with no drift in time, and with the same degree of accuracy as is the case with any reversible dye system except for the fact that F has to be replaced by $2F$. At any event for this paper only such cases were selected in which the experiments showed a strict validity of that statement. The strict fulfilment of this condition implied a careful selection of the material and showed a relative scarcity of systems worthy of a report on the potentiometric findings.

In many cases, such as in the case of tartaric acid, the oxidation of the ferrous complex not only conveys an oxidation of the iron, but the oxidized complex is unstable; an intramolecular rearrangement takes place in that the organic part of the complex undergoes oxidation at the expense of the ferric iron. These are cases where iron works as a catalyst for the oxidation of organic substances when an otherwise inert oxidant is used such as O_2. In such a case there will be a drift of potential with time. It seemed appropriate to avoid such cases in this paper, with the prospect of dealing with them separately later on. All anions used as complex formers in this paper will be such as are not liable to a catalytic oxidation with iron as catalyst, or at least to be stable over a reasonable period of time. In this sense, some of the organic acids, containing only carboxyl groups, could be included in this paper whereas hydroxy acids were omitted because of the ease of their oxidation under the influence of iron. The number of complex-forming anions suitable for the purpose is rather restricted under these circumstances yet will be sufficient to demonstrate the essential features of the problem.

Methods

The method used was the potentiometric titration of the reduced complex by a suitable oxidant, or the titration of the oxidized complex by a suitable reductant. Neither the oxidant nor the reductant used was a complex former of any affinity comparable with the affinity of the complex former to be studied.

The oxidative titration has to start with the completely reduced complex. It is very difficult to obtain the complex in an entirely reduced state directly by simply dissolving a plain ferrous salt (say $FeSO_4$) in an excess of the complex former, say pyrophosphate.

The reason for this difficulty is the ease with which these ferrous complexes are usually oxidized even with traces of oxygen. For this reason, pH of the solution of, say, ferrous pyrophosphate cannot be determined by means of the ordinary hydrogen electrode. We attempted to reduce the complex completely by means of colloidal palladium and hydrogen according to the method described by Michaelis and Eagle (7). In fact, the reduction could be brought about by this method so completely that a platinized electrode in hydrogen gave a stable and trustworthy reading for calculation of pH. The reduction, however, required an unusually long time and was not practicable. When, however, a small trace of a reversible dye of sufficiently negative potential range was added, such as safranine, rosinduline,[2] anthraquinone-β-sulfonate, or gallophenine, the reduction by means of palladium plus hydrogen went on very quickly as though catalyzed by the dye. Dyes of less negative potential ranges, such as methylene blue or the indophenols, were very much less, or not at all, efficient in this respect. After the completion of the reduction and the determination of pH by means of the platinized electrode, the hydrogen was removed by pure N_2 and the oxidation titration started as shown in the quoted paper (7). In suitable cases quinone or phenolindophenol was used as oxidant. These oxidants could be used only when the range of titration was restricted to a sufficiently negative range of the potential.

Much freer from these restrictions was the reductive titration of the ferric complex. Any leucodye of sufficiently negative potential range could be used as reductant. We used rosinduline, indigo di- or tetrasulfonate, and some others. • The dyes were reduced in purely aqueous solution without buffer by means of hydrogen and a trace of colloidal palladium, and in this form poured into the burette, the titration being performed in a stream of pure nitrogen. The potentials thus read at blank electrodes were instantaneously established, steady, reproducible, and identical at different electrodes just as well as in any strictly reversible dyestuff system.

Several attempts were also made to obtain titration curves by titrating the ferric compound with sodium hydrosulfite. The

[2] This useful dye will be described in a subsequent paper.

experiences gained on this occasion seem worth mentioning. When ferric pyrophosphate is titrated with hydrosulfite, no reliable potential reading can be obtained. The potentials are neither stable nor reproducible with different blank electrodes. When however a small trace of a reversible dyestuff is added to the solution the potentials become stable and reproducible over that potential range covered by the dye. Only with a dye of sufficiently negative range of potential, such as rosinduline, was the titration curve reproducible and definite approximately to the end of the titration of the iron-pyrophosphate systems. Anyhow, no
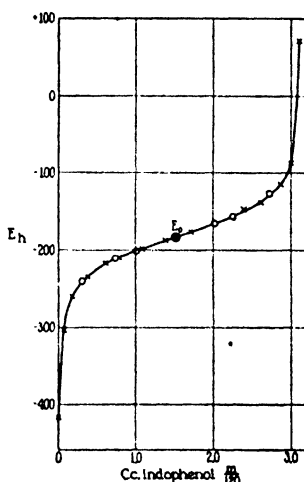
Fig. 1. Ferropyrophosphate is titrated with phenolindophenol as oxidant, at pH 8.068, at 30°. Abscissa, cc. of oxidant added; ordinate, potential referred to the normal hydrogen electrode.

titration curves obtained with hydrosulfite were used for the definite values as plotted in Fig. 2.

Results

The results are shown in the following graphs. One example of a titration curve is presented in Fig. 1.

Fig. 1 shows the oxidation of ferropyrophosphate by phenolindophenol, at 30°. 20 cc. of a 0.9 M solution of sodium pyrophosphate are mixed with a small amount of 0.10 N HCl to fix the pH. A small amount of $FeCl_3$ is added. After addition of a very small

amount of phenosafranine and colloidal palladium (the commercial preparation according to Nacht-Pahl, dissolved 1:1000) the mixture is reduced in the electrode vessel by a stream of hydrogen gas. By means of a platinized electrode the pH is
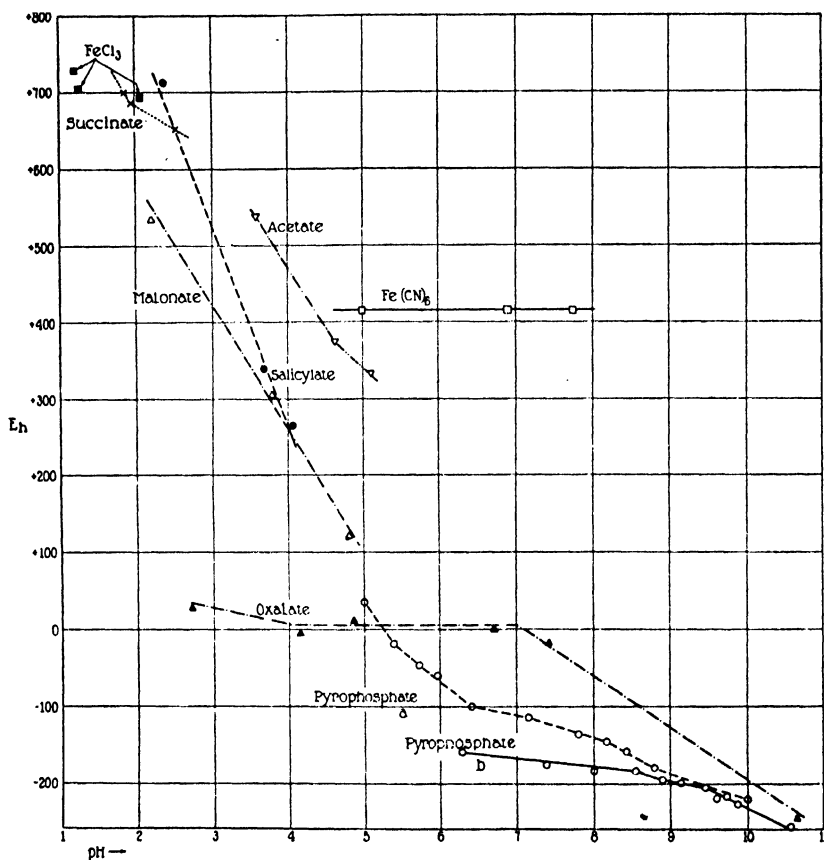
FIG. 2. The normal potential of various iron complexes at varied pH, referred to the normal hydrogen electrode, at 30°. Abscissa, pH; ordinate, normal potential.

found to be 8.008. After finishing the whole titration with the oxidant the reduction by hydrogen is started over again, and pH is found 8.128. So the average, 8.068, is considered as the mean value of pH during the titration.

The abscissa shows cc. of $\frac{M}{120}$ phenolindophenol added. The ordinates show the potential in millivolts, referred to a saturated calomel electrode the potential of which was $+0.442$ volt referred to the normal hydrogen electrode.

The crosses are the values observed, circles values calculated to construct the curve $E = E_0 + 0.0601 \log \frac{x}{a - x}$.

All potentials shown in Fig. 2 are based on titration curves of the same type and accuracy as this example, shown in Fig. 1.

Fig. 2 gives a graph of the normal potential of various iron complex compounds at varied pH.

Each point of this graph is the result of an individual complete titration curve such as shown in Fig. 1, and represents the normal potential E_0, *i.e.* the potential of a 50:50 mixture of the ferro and ferri compound. The abscissa plots pH and the ordinate, potential referred to the normal hydrogen electrode. Each complex was studied over such a range of pH as the solubility of the complex concerned permitted. Thus, the right-hand end of each curve shows approximately the highest pH at which no precipitation of a hydroxide or basic salt of iron occurred.

The pH was usually fixed by the buffering action of the complex-forming anion, which was always present in high excess to the iron, and was modified by addition of HCl or NaOH.

For the $FeCl_3$-$FeCl_2$ system three points, obtained under different conditions of concentrations and therefore not quite comparable with each other, are plotted. Therefore they are not connected to a curve but drawn separately to show the potential range of such a system, for comparison.

The curves for ferricyanide-ferrocyanide are taken from previous experiments (8) and refer to a solution 0.001 M with respect to each of the constituents in a 0.05 M solution of KCl.

Succinate—A mixture of 20 cc. of 0.1 M solution of sodium succinate and 1 cc. of 0.05 M ferri-ammonium sulfate is titrated with 0.0125 M reduced indigo tetrasulfonate.

Salicylate—A mixture of 10 cc. of 0.05 M sodium salicylate and 1 cc. of 0.05 M ferri-ammonium sulfate is titrated with 1 per cent reduced rosinduline.

Malonate—A mixture of 10 cc. of 0.1 M malonic acid and 10 cc.

of dilute NaOH or buffer and 1 cc. of 0.05 M ferri-ammonium sulfate is titrated with 1 per cent reduced rosinduline.

Oxalate—A mixture of 10 cc. of 2 M potassium oxalate and 10 cc. of buffer and 1 cc. of 0.05 M ferri-ammonium sulfate is titrated with reduced rosinduline.

Acetate—Standard acetate 20 cc., ferri-ammonium sulfate 1 cc. of 0.05 M, and acetic acid or NaOH accordingly, were titrated with reduced indigo tetrasulfonate.

Pyrophosphate—20 cc. of $\frac{M}{9}$ sodium pyrophosphate + 0.2 cc. of 0.05 M ferri-ammonium sulfate (upper curve) and + 0.8 cc. of 0.05 M ferri-ammonium sulfate (lower curve) reduced with palladium-H_2 and a trace of safranine as catalyst, titrated partially with phenolindophenol, and partially with ferricyanide were used.

SUMMARY

The most important features of Fig. 2 may be summarized in this way:

The normal potential range of ferro-ferri systems may lie between very wide limits, from $+0.7$ to -0.25 volts, according to the chemical nature of the complex-forming anion. The dependence of such a potential on pH is very small in some cases, but extremely great in others. It is negligible for ferrocyanide, and over a certain range of pH also for oxalate and for pyrophosphate. It is extremely great for acetate, salicylate, malonate, indeed for all such complexes in which the affinity of iron for the complex-forming anion is comparable to that of the hydroxyl ion. In agreement herewith, complexes of this kind are stable only in slightly acid solution, whereas in alkaline solution precipitation of iron hydroxide or insoluble basic salts occurs. These curves can, therefore, be traced only to a pH 5 or so.

On comparing oxalate, malonate, and succinate the difference in the behavior of these representatives of a homologous series is striking. In oxalate the iron atom is part of a five-membered ring, in malonate a six-membered, and in succinate a seven-membered ring. Accordingly, the stability of the complex with increasing pH and the potential range of the complex is widely different in these three cases.

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POTENTIOMETRIC STUDY OF PYOCYANINE

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Bacillus pyocyaneus produces, according to the conditions, different varieties of pigment of which pyocyanine is best known and easily isolated because of its solubility in chloroform. This pigment is indigo blue at alkaline reaction, wine-red at acid reaction, and is reducible to a leuco compound by various reductants or in the absence of air by the living bacillus itself, and the leucodye is readily reoxidized on exposure to the air. It behaves in this respect as any of the reversible organic dyestuffs of the quinone type. The pigment can easily be obtained in a pure crystallized form and has been chemically studied by Ledderhose (1), MacCombie and Scarborough (2), and more recently by Wrede and Strack (3). The latter authors consider it as a methylated oxyphenazine and confirmed this assumption by a synthesis. From freezing point determination in glacial acetic acid they conclude that the oxidized form of the dye has double the molecular weight of the reduced form. If this be true also for aqueous solutions, the potential curve obtained on titrating the reduced dye with an oxidant or the oxidized dye with a reductant should show a form considerably different from the one of an ordinary organic dyestuff of the quinone type. In our potentiometric studies, however, we found no difference in the potential curve from the one of an ordinary dye. This at least holds for neutral and alkaline solutions. To be sure in acid solution there is a striking difference, but not of such a nature as to account for a bimolecular structure. This will be discussed later on.

Once the reversible nature of the oxidation and reduction of pyocyanine has been recognized it was suggestive to undertake a

potentiometric study of this pigment.   This is the task of the present paper.

Cultures of the bacillus on peptone glycerol agar were extracted by chloroform after a sufficient amount of the pigment had been formed.   The pigment was purified by extracting the chloroform solution by an acid aqueous solution, this made alkaline, and again extracted with chloroform, and this process repeated several times.   Finally the chloroform solution was allowed to evaporate and the crystalline blue dye was dissolved in water and mixed with the various buffers according to the needs.

The dye is blue at alkaline reaction and wine-red at acid reaction. The pH at which the color is a 50 per cent mixture of the blue and the red form was found to be 4.9, by comparing in a Walpole comparator an optical mixture of the separated blue and the separated red form (in NaOH respectively HCl) with a true mixture of the two forms established by an acetate buffer of suitable pH. When the alkaline blue solution is reduced, say by a trace of colloidal palladium in a stream of hydrogen, the color vanishes and is reestablished on exposure to the air.

. If, however, the acid red form of the dye is reduced there is an intermediary green stage before the complete decoloration.   In the same manner when the completely reduced colorless dye is titrated with a colorless oxidant (such as ferricyanide, which in the concentration used shows no noticeable color) in an alkaline solution, the colorless leucodye directly turns blue, whereas in an acid solution the leucodye first turns green and just when the half of the equivalent amount of the oxidant is being exceeded (judged from the potentiometric end-point of titration) begins to turn red. Hence it may be concluded that the chemical process of oxidation or reduction is a simple one step reaction at pH say from 6 to 10, but a two step reaction in acid ranges.

The amount of oxidant necessary to oxidize the completely reduced colorless state to the fully oxidized one is the same whether the oxidation leads directly to the blue compound or in two steps to the red compound.   The amount of oxidant necessary to oxidize the colorless reduced state in an acid solution to the green intermediary form is precisely half the above amount.

If pH is very high, around 12, the blue oxidized form, on addition of a further excess of oxidant (ferricyanide) is very gradually and irreversibly oxidized to a colorless compound.   For this

reason the end-point of titration of the reversible part of the oxidation is not very sharp at pH 12. At least after the end-point of titration has been reached the potentials gradually drift back to the negative side. Yet, even under these circumstances the end-point of the reversible oxidation process can be determined with a practically satisfactory approximation.   ·

The same methods were used for the potentiometric titration as described in a previous paper (4). In part the dye was titrated by a reductant such as a reduced organic leucodye of sufficiently negative range of potential. A very suitable leucodye for this purpose was rosinduline, a dye closely related to the safranines. Its potential range is very negative and its reversibility very satisfactory, this statement implying that the leucodye is a stable compound even in strongly alkaline solutions. In other experiments the pyocyanine, dissolved in a buffer solution, was completely reduced in the electrode vessel by means of a trace of colloidal palladium and hydrogen gas, thereafter the hydrogen completely washed out by pure nitrogen, and the reduced pyocyanine was titrated with potassium ferricyanide. In both methods the pH of the well buffered solution was determined by means of a platinized electrode in the completely reduced state of the system in a hydrogen atmosphere.

The concentration of the ferricyanide used for titration was 0.001 or 0.002 M, and 1 to 3 cc. were used to the titration. Despite the low concentration the potentials were established practically instantaneously and were stable in time, independent of shaking and of the individuality of the blank platinum electrodes, just as well as in any of the well studied organic dyestuff systems. Only at the very beginning and the very end of the titration was the stability and reproducibility less, a phenomenon usually encountered even in the best of reversible systems.

At pH from 6 to 12 the titration curve was in every respect analogous to the one for reversible organic dyes. This observation leaves no doubt that the oxidation reduction process can be symbolized by such a formula as

$$X + 2H \rightleftharpoons XH_2$$

or

$$X + 2H^+ + 2\,\epsilon \rightleftharpoons XH_2$$
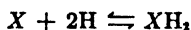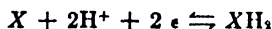
In acid solutions things become more complicated. First of all the color of the aqueous solution is red instead of blue, obviously due to the basic property of one of the N atoms. When the reduced colorless form in an acid solution is titrated with an oxidant the total amount of the oxidant to the end-point of titration is the same as at alkaline reaction, but the course of the potential curve is quite different from the other. First of all the curve is much steeper. In the second place there is a distinct step precisely when half the amount of the total oxidant has been added. At the same point a change in color takes place. In the first half of the titration the previously colorless solution gradually turns more and more yellowish green. After exceeding half the equivalent of the oxidant suddenly the red tint appears leading at the end of the titration to the pure wine-red shade of pyocyanine as it usually appears in an acid solution.

There are different possibilities to account for the two step oxidation. First we may assume that the supposedly pure dye is really a mixture of two dyes, each with a definite potential range. The normal potentials of the two dyes might by chance be equal to each other at alkaline reaction but become more and more different with increasing pH. Such an assumption is however very unlikely, as we consider the ease of crystallizability of the dye and the reproducibility of the results with different independent preparations of the dye. It is furthermore incompatible with the fact that in acid solution the color first turns green, then red. No tint whatever, in admixture to the yellowish green of the first step, can produce the red tint of the second step, and it is evident that the red arises from the green by oxidation.

There is left only the assumption that one chemical individual is oxidized in two steps. Such a hypothesis is open to two different interpretations. Either we may think the intermediary product to be a meriquinone, as this concept has been defined by Willstätter and Piccard (5), a molecular compound of the oxidized and the reduced form. Or we may think of a stepwise oxidation of the reduced dye in such a way that 1 molecule of the reduced form detaches the 2 hydrogen atoms at acid reaction in two successive steps, each concerning one, whereas at alkaline reaction the ordinary type of oxidation of organic quinoid compounds takes place in that both hydrogen atoms are detached at the same time.

A decision as to the validity of one or the other of these two assumptions may be attempted by an analysis of the titration curve. If a meriquinone be formed the two steps can be formulated.

(1) $\qquad\qquad 2\ XH_2 \rightarrow (XH_2,\ X) + 2\ H$

I

(2) $\qquad\qquad (XH_2,\ X) \rightarrow 2\ X + 2\ H$
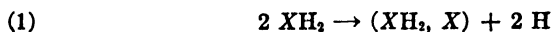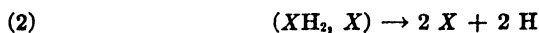
If the other mechanism be valid the two steps are these

(1) $\qquad\qquad XH_2 \rightarrow XH + H$

II

(2) $\qquad\qquad XH \rightarrow X + H$

In the case of meriquinone formation the titration curve to be expected is very complicated. We may refer to a paper by Clark, Cohen, and Gibbs on meriquinone-forming organic compounds (6), but we may save ourselves the trouble of a detailed discussion here and merely emphasize the fact that the shape of such a curve is distinctly different from the ordinary one. We may add that all the known meriquinones are very intense in color whereas the intermediary step in the present case shows a rather pale color. In contrast to that, in the other case the shape of the titration curve would be very simple provided the two steps are sufficiently separated to prevent overlapping of the two halves of the curves. At pH 1 to 3 the two steps are fortunately so well separated that no noticeable overlapping can take place. Here each half of the titration curve agrees perfectly with the formulation

$$E = E_0 + 0.06 \log \frac{\text{oxidized form}}{\text{reduced form}}$$

E_0 being one definite value for the first step and another one for the second step. So we prefer at the present time the second interpretation and come to the following conclusion. In very acid solution the reduced dye is oxidized in two distinct steps, at each losing 1 hydrogen atom. Either of these two steps shows a titration curve completely analogous to the ferrocyanide-ferricyanide system with a slope of 0.06 volt for each power of 10 of the ratio of oxidized form to reduced form.

At higher pH the end-point of the first step and the beginning of the second overlap and do so the more the higher the pH.   From pH about 6 on, the overlapping is complete or, in other words, the titration curve becomes like that of an ordinary organic dye with a slope of 0.03 instead of 0.06 volt.

At very high pH, about 12, another complication arises which has a somewhat different aspect according to whether the reduced dye is titrated with an oxidant or the oxidized dye is titrated with a reductant.   If the reduced dye is titrated with ferricyanide the curve is the expected one, until almost the end of the titration. When this end is reached and the potential is expected to jump into the range of the ferricyanide-ferrocyanide system the potentials become unsteady, drifting gradually back to values more negative than would correspond to the range of the oxidant system. At the same time the color of the dye gradually fades out.   Obviously an irreversible progressive oxidation of the oxidized blue form to a colorless compound takes place.   On the other hand when the oxidized dye is titrated with a reductant, even before the addition of the reductant the potential is more negative than would be expected from a solution containing only the oxidized state of the dye.   This phenomenon is due to the same property of the dye as the other.   The tendency of the oxidized dye to undergo an irreversible oxidative destruction in strongly alkaline solutions manifests itself in the absence of an oxidant by the establishment of an unexpectedly negative potential.   But, disregarding the very beginning of the titration curve, the rest of the curve is otherwise regular throughout even in strongly alkaline solutions.

The tendency of the dye to undergo an irreversible oxidation in very alkaline solution has been already observed by Wrede and Strack.   They found that the blue dye in 0.1 N NaOH gradually turns reddish on consumption of oxygen from the air, or if no air is present, in such a way that a part of the dye is oxidized at the expense of another part which is reduced.   .

When we plot the potentials of the half reduced dye at varied pH there is no difficulty in doing so at pH greater than 6.   These potentials lie all on a straight line with a slope of 0.06 volt per unit of pH, except for the fact that at very high pH a flattening of the slope begins to take place.   Applying the principle developed by Clark and Cohen (7) we might interpret this flattening as due to

a dissociation constant of the reduced form. The value of this constant may be estimated by a graphic interpolation pK = 10.2 and is likely that of a phenolic group in the reductant which is missing in the oxidized form.

As we attempt to extend this plotting of the curve toward the acid side we have to plot the normal potentials for each of the two
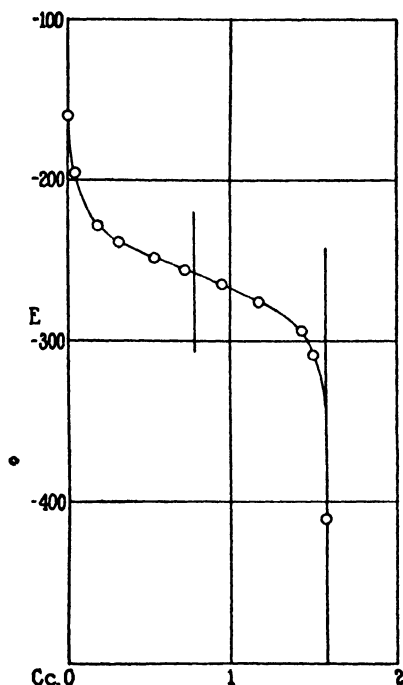
Fig. 1. Pyôcyanine titrated with leucorosinduline at pH 6.7$\overset{4}{4}$ and 30°. Abscissa, cc. of the leucodye; ordinate, potential referred to the saturated calomel electrode.

steps separately. This is easy at pH 1 to 3 where the two steps do not overlap. Between pH 4 and 5.5 the computation of what we may call the normal potential for each step is somewhat impaired due to the overlapping of the two steps, but the ambiguity due to the rather arbitrary graphic interpolation is relatively small. By such an interpretation we may see that the curve branches into two curves from pH about 4.9. This is precisely that pH where, in the oxidized state, the blue color begins to turn red.

The shape of these two branches of the curve is surprisingly simple. One branch is an accurate extension of the rectilinear course as it is at pH greater than 5, and the other is a bend resulting in a horizontal straight line.

Fig. 1 shows the reduction of pyocyanine by leucorosinduline, at pH 6.74 (phosphate buffer). The abscissa measures cc. of leucorosinduline added; the ordinate, potential referred to a saturated calomel electrode. Titration took place in a constant temperature room at 30°. The drawn out curve is the one calcu-
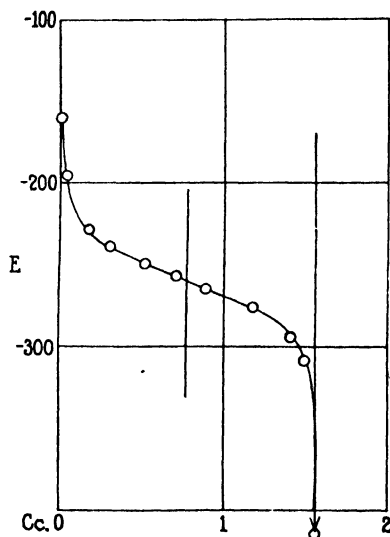
Fɪɢ. 2. The same as in Fig. 1 except for pH 7.25 (phosphate buffer)

lated from $E = E_0 + 0.030 \log \dfrac{x}{a - x}$ where a is the amount of reductant used up at the end-point of titration, and x is the amount of reductant indicated on the abscissa.

The potential of the calomel electrode used, at 30°, was $+ 0.442$ volt with respect to the normal hydrogen electrode. The end-point and the mid-point of the titration are indicated by perpendicular lines.

Similarly Figs. 2–4 were plotted but at different pH values: Fig. 2 for pH 7.25 (phosphate buffer); Fig. 3 for pH 8.407 (veronal buffer) (8); Fig. 4 for pH 11.888 (buffer of secondary phosphate +
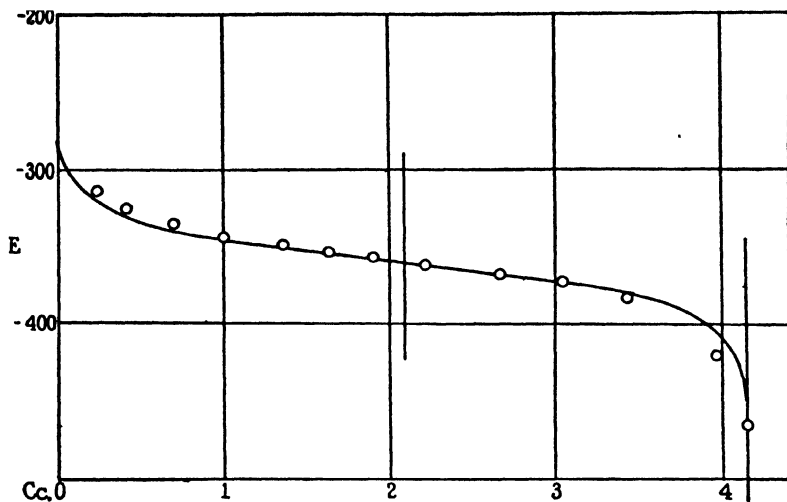
FIG. 3. The same as in Fig. 1 except for pH 8.407 (veronal buffer)
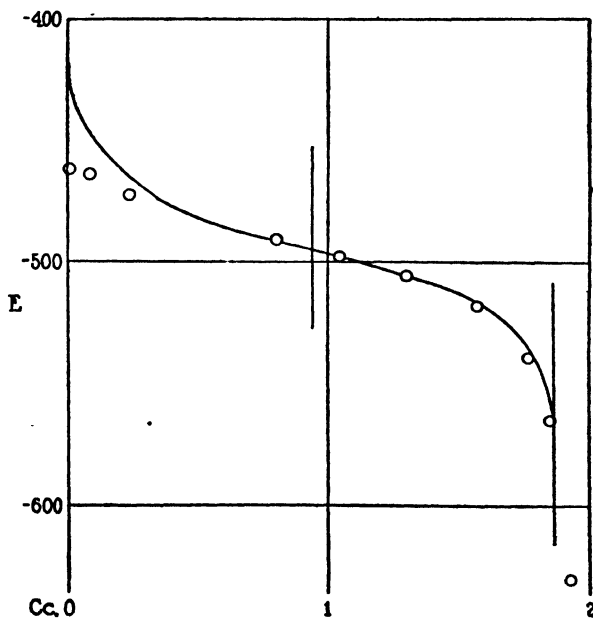
FIG. 4. The same as in Fig. 1 except for pH 11.888 (buffer of secondary phosphate + NaOH).

NaOH).   At such a high pH the potentials at the beginning are more negative than expected from the calculated curve, but later fit the calculated curve.

Fig. 5 gives the values obtained when reduced pyocyanine is titrated with 0.001 M potassium ferricyanide.   The calculated, drawn out curve is composed of two halves each following the
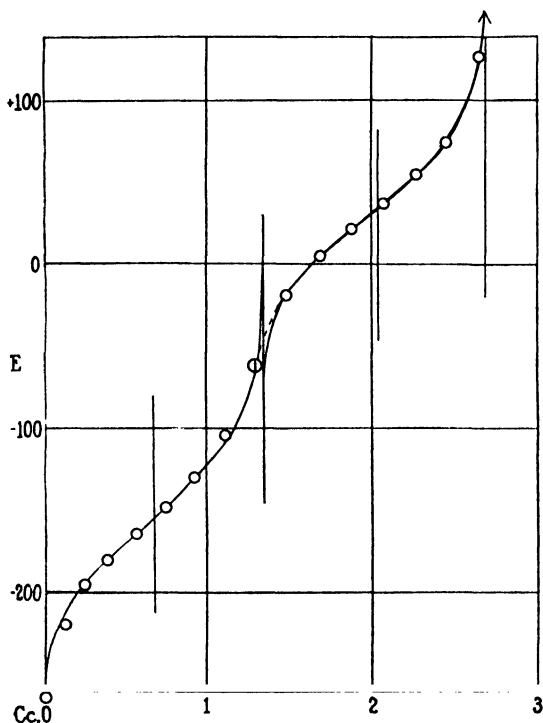
Fig. 5. Reduced pyocyanine is titrated with potassium ferricyanide, at pH 1.82.   The significance of the ordinate and the abscissa is the same as in Fig. 1.

formula $E = E_0 + 0.060 \log \dfrac{x}{a - x}$ where a is the total amount of oxidant required for each of the two steps of oxidation, and x is the amount of oxidant taken from the zero point of each step.   The dotted part of the curve is a graphic interpolation in order to smoothen the overlapping part of the two curves.   pH = 1.82 (HCl + KCl).

Fig. 6 gives results on the same substance as does Fig. 5 but for pH 3.045 (acetic acid). Here the separation of the two steps is less pronounced, yet, by no means, is the curve comparable to the one shown in Figs. 1, 2, or 3, for, if one would try to take the whole curve as a single step, and smoothen the middle part correspondingly so as to fit such a simple assumption, the slope of the curve would be double that in Figs. 3, 4, or 5.
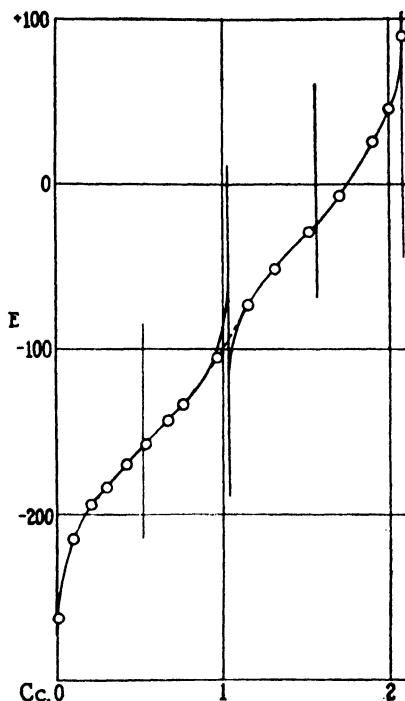
FIG. 6. The same as in Fig. 5 except for pH 3.045

All examples of titration curves in alkaline solutions shown in Figs. 1–3 are reductive titrations. The oxidative curves (with ferricyanide as oxidant) were not quite so perfectly fitting to the theoretical course as, in general, they were somewhat too steep, very little indeed, but probably outside the experimental errors. This we believe to be due to the fact that the addition of an ion of such a high valence as ferrocyanide causes an appreciable change in ionic strength. The deviations were, however, very small and did

not prevent the graphic determination of the normal potentials within 1 or 2 millivolts. The points plotted in Fig. 7 are, in fact, taken from both oxidative and reductive titration curves. At low pH, only oxidative titration curves could be used for the reason mentioned above.

Fig. 7 is a summary of all titration curves of which Figs. 1–6 are examples. The abscissa measures pH; the ordinate, normal potential (*i.e.* potential of the mid-point of titration) referred to the calomel electrode, in millivolts. Add 242 millivolts to obtain the potential referred to the normal hydrogen electrode.
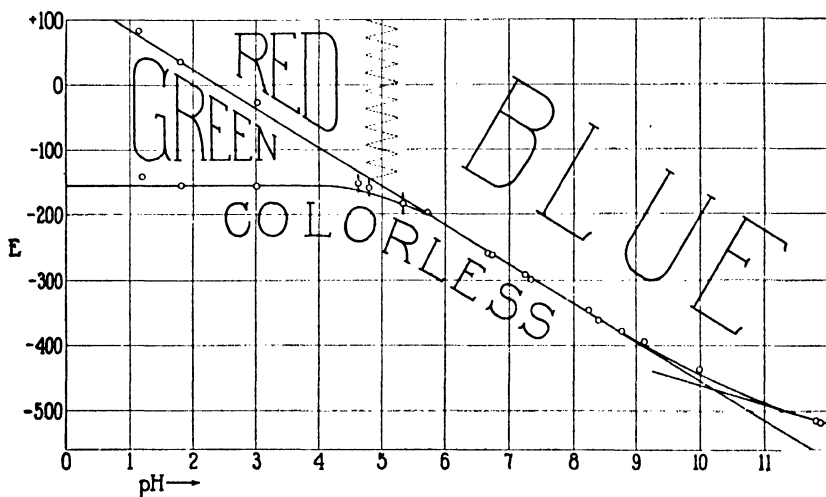
Fig. 7. Normal potential of pyocyanine at varied pH, at 30°, referred to the saturated calomel electrode, in millivolts. (Add 242 millivolts to obtain the potential referred to the normal hydrogen electrode.)

Between pH 6 and 9 the slope is 0.060 volt per pH unit. With increasing pH, the curve flattens. The point of intersection of the 0.06 slope and a straight line of 0.03 slope forming the best fitting tangent to the curve, is supposed to indicate a dissociation constant (pK = 10.2 approximately) of the reduced form, probably due to the phenol group.

To the left-hand side from pH 6, the curve is split into two branches, according to the two steps of oxidation. One branch is a straight continuation of the curve, the other branches off horizontally.

The three points (around pH 5) marked with arrows have the following significance. Here the two steps of oxidation (corresponding to those in Fig. 5) overlap so much that the two constants cannot be determined separately. But the slope of the titration was much steeper than for higher pH. So the mid-point potential was taken and the arrows indicate that this value is the average of two values, one being a little higher, the other a little lower, so as to fit the branching of the theoretical curve.

The color of pyocyanine is determined both by pH and the potential. The field below the curve is colorless, the field above is

TABLE I

Normal Potential (i.e. Potential in Half Reduced State, Referred to Normal Hydrogen Electrode) at 30°, in Millivolts, for Pyocyanine, as Interpolated from Fig. 7

pH	First step	Second step	For comparison (according to Clark and associates)	
			Methylene blue	Indigo tetrasulfonate
3	+207	+97		
4	+146	+68		
5	+86	+78		
6		+26	+47	+6
7		−34	+11	−46
8		−93	−50	−108
9		−149		
10		−198		
11		−246		

red or blue, according to the pH, with a transition zone around pH 4.9. This point is the beginning of the branching. The wedge produced by the branching encloses the green state of the dye. (It may be added that this green state of pyocyanine has nothing to do with the fluorescent yellowish green pigment sometimes produced by *Bacillus pyocyaneus* on such media as will not cause it to produce the blue dye. The fluorescent green dye is different from the ordinary blue dye. It differs in that it cannot be extracted by chloroform, and so can be separated easily from the blue pigment.)

SUMMARY

The blue pigment of *Bacillus pyocyaneus* is reversibly oxidizable and reducible. In ranges of pH > 6 it behaves entirely as a reversible dye of a quinoid structure. The slope of the titration curve indicates that 1 molecule of the dye combines with 2 H atoms simultaneously. At pH ranges < 6 the titration curves show a different shape which can be interpreted by the assumption that the 2 H atoms are accepted in two separate steps. The existence of these two steps of reduction is confirmed by the fact that in acid reaction the color, on reduction, changes in two different steps, red → green → colorless, whereas at alkaline reaction the color change takes place in one step, blue → colorless. The normal potentials of the dye at various pH are summarized in Fig. 7. It can be seen from Table I that around pH 7 the potential range of this dye lies between that of methylene blue and indigo tetrasulfonate.

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ROSINDULINE AS OXIDATION-REDUCTION INDICATOR

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In previous communications (1) the dyestuff rosinduline was mentioned with the promise that a full description would be forthcoming later. Its properties make it a particularly desirable addition to the list of reversibly oxidizable and reducible dyestuffs available as indicators for oxidation-reduction potentials in very negative potential ranges, and warrant its singling out for presentation in this paper.

The dye, Rosinduline 2 G (Colour Index No. 830, or Schultz' Farbstofftabellen No. 674, synthesized by Hepp in 1890) (2) is the sodium salt of a monosulfonic acid of the compound shown in the formula (called Rosindon). The free acid can be precipitated
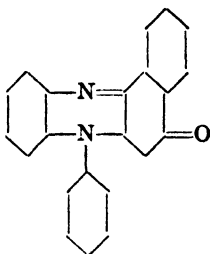
by HCl and crystallizes readily. The sulfonic acid group is in one of the benzene rings; it is unknown in which. The dye has an intense scarlet red color; the leucodye in higher concentrations is slightly yellow brown, but colorless in the lower concentrations in which the dye may be used as an indicator. The dye is easily soluble and stable both in the oxidized and reduced state even in very strongly alkaline solution and so differs, advantageously, I believe, from all dyes of a comparably negative potential range

such as are recommended by Rapkine, Struyck, and Wurmser (3) and Wurmser and Geloso (4). All those dyes suffer in part from low solubility in certain pH ranges, inclination to enter into a colloidal state, instability either in the reduced or the oxidized form, or from weakness in color (the anthraquinone sulfonates investigated by Conant and Fieser (5)). Moreover, the potential range of this dye is even more negative than is that of any of the others. The potentials are perfectly reproducible and steady even at very high pH so that this dye may be used for class experiments for reversible oxidation-reduction titration curves.

The dye can be reduced by colloidal palladium and hydrogen; the leucodye is easily soluble and very stable, except of course for its sensitivity to oxygen, and suitable to serve as a convenient reductant for titration instead of sodium hydrosulfite or titanous salts. There will be only a few cases in which the potential range is not negative enough for such a purpose.

The normal potential range of the dye was determined by the method described in a previous paper (6) for various pH, by reducing the buffered solution of the dye with palladium and hydrogen, measuring pH with a platinized electrode, replacing N_2 for H_2, titrating with quinone, and reading the potentials at blank electrodes. The titration curves were throughout regular and fitted perfectly the course of a theoretically constructed curve for dyestuffs of quinoide type. The dye is easily available in pure condition. There was at my disposal also a specimen of a similar dye with the sulfonic acid group in the naphthalene nucleus (Rosinduline G, Colour Index No. 831).[1] Its potentials were scarcely distinguishable from those of the other.

No individual titration experiments will be shown as the titration curves were perfect and unambiguous[2] and only a summary of the results is plotted in Fig. 1, in which there is shown the potential of half reduced Rosinduline 2 G at 30° (at the ordinate),

[1] At my request these dyes were prepared and placed at my disposal by the I. G. Farbenindustrie, Leverkusen, Germany. My best thanks go to this corporation.

[2] This is very much in contrast to the curves obtained with a preparation of safranine at my disposal (Colour Index No. 841, or Schultz' Farbstofftabellen No. 679).

referred to the normal hydrogen electrode, at varied pH (in the abscissa). Acetate, primary plus secondary phosphate, veronal (7)
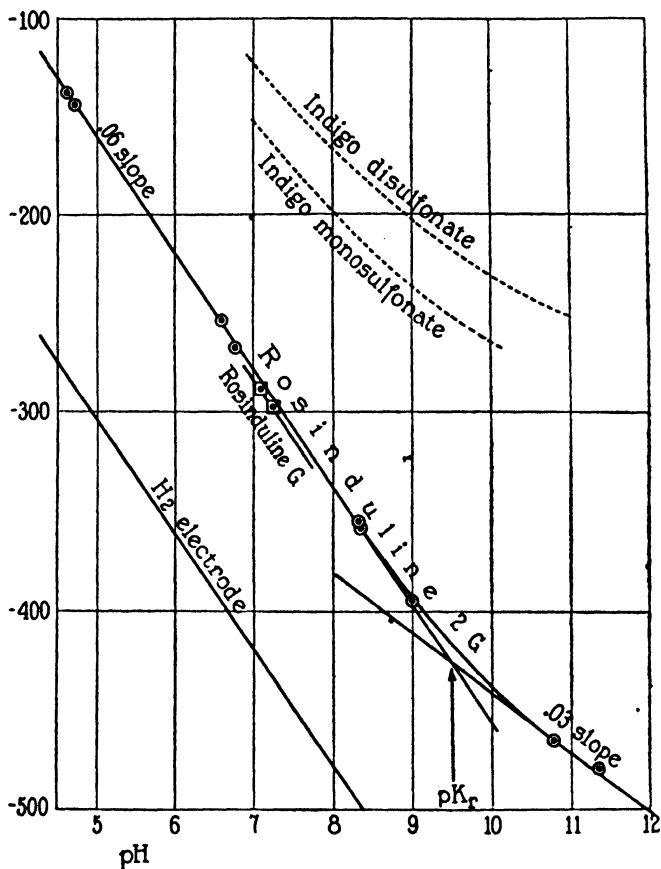
FIG. 1. Potential of Rosinduline 2 G, in the half reduced state, at varied pH, referred to the normal hydrogen electrode, at 30°. The two points marked □ refer to Rosinduline G which differs from the other only by the position of the sulfonic acid group. A part of the curves for indigo disulfohate and indigo monosulfonate drawn according to the measurement of Clark, Sullivan, and Cohen (8), is shown as comparison for the potential range covered by the dye. The abscissa measures pH; the ordinate, potential.

and secondary phosphate plus NaOH were used as buffers. The two points marked by squares refer to Rosinduline G (sul-

fonated in the naphthaline nucleus instead of the benzene nucleus). The dissociation constant of the reduced form of the dye as indicated by the change of a 0.06 slope to a 0.03 slope at pH 9.5 is obviously that of the phenolic hydroxyl group present in the reduced form but missing in the oxidized form.

The two upper curves, for comparison, are those for indigo disulfonate and indigo monosulfonate according to Clark, Sullivan, and Cohen (8). It is worth mentioning that in the pH range 8 to 9, in which the veronal buffer (7) was used, no such irregularities were observed as Clark, Sullivan, and Cohen sometimes encountered by using the borate buffer for the indigo dyes.

SUMMARY

Rosinduline is recommended as an indictor for oxidation-reduction potential in a very negative potential range. Its normal potential at 30°, referred to the normal hydrogen electrode is

pH	5	6	7	8	9	10	11
Normal potential, *volts*	−0.161	−0.221	−0.281	−0.340	−0.395	−0.438	−0.480

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THE EFFECT OF INGESTED UREA ON NITROGEN
METABOLISM

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In studies of the nitrogen metabolism of patients with nephritis,
it was found that whenever urea was given for diuretic purposes,
large positive nitrogen balances developed. The urea was not
quantitatively recovered during the period of urea administration
nor in the subsequent period.[1] Although the results of these stud-
ies on nine subjects were so uniform and consistent as to leave little
doubt as to the accuracy of the observation, it was recognized that
no work with hospital patients could achieve the accuracy attain-
able with interested and intelligent normal subjects. Moreover, it
was hoped that experiments with such normal persons might throw
some light upon the fate of the lost nitrogen. Therefore, four
experiments, which are described below, were conducted upon three
normal subjects. The results of the clinical experiments were
confirmed, but no explanation for the anomalous facts has been
discovered.

HISTORICAL

In both physiologic and clinical studies of renal function it has
been generally assumed that urea is an end-product of protein
metabolism that cannot be utilized by the organism and must be
excreted in the urine. Nevertheless it has been claimed, on the
basis of animal experiments, that both urea and ammonia salts
can serve as partial substitutes for protein in the diet of animals.

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[1] A preliminary report of the studies on five of these patients and one
normal was presented before the American Society for Clinical Investi-
gation in April, 1928.

The subject has been a source of controversy, which has been admirably reviewed by Cathcart (1) and by Mitchell and Hamilton (2).

The effects of urea administration in man have been less extensively studied and never, as far as the authors know, for the purpose of learning its effect on nitrogen metabolism.

Available data deal entirely with experiments on renal function. Janney (3) determined the urinary nitrogen of eight patients for a number of days preceding and following a single dose of 20 gm. of urea. In six of the eight cases the extra nitrogen excreted after the urea fell short of the quantity given. In one of the remaining two cases, No. 19, a profuse diuresis may have accounted for the sweeping out of about 24 gm. of excess nitrogen. Cathcart and Green (4) kept a normal adult on a potato and butter diet for 6 days. On the 5th day they administered 12 gm. of urea, of which they recovered 95 per cent in the next 48 hours. Addis and Watanabe (5) studied the rate of the urea excretion of thirty normal adults. These subjects received for 6 successive days a diet containing 75 gm. of protein, 120 gm. of carbohydrate, and 220 gm. of fat with 1710 cc. of fluid. On the last 3 days one group was given 20 gm. of urea daily and another group 40 gm., while a third group served as controls without urea. Urine was analyzed daily for urea nitrogen only. Urea nitrogen excreted in the last 3 days in excess of the average amount excreted on the 2nd and 3rd days was assumed to have been derived from the administered urea. The average urine urea excreted by the control subjects on the last 3 days agreed remarkably closely with that of the 2nd and 3rd days. A fraction of the ingested urea nitrogen was not recovered during the experimental period, the fraction being approximately the same whether 20 or 40 gm. were given. Addis and Watanabe state, without publishing further data, that the few gm. of nitrogen retained could be accounted for if the experiment was continued for an extra day. O'Hare (6) applied to patients the urea and salt test of von Monakow. The patients were given daily 75 gm. of protein, 4 gm. of sodium chloride, 1500 cc. of water, and enough fat and carbohydrate to bring the food value of the diet to 2000 to 2200 calories. After fluid, salt, and nitrogen equilibrium had been attained, 20 gm. of urea were given. During the test the volume and nitrogen content of the urine were determined daily. Analysis of his results, by the method applied to those of Janney above, discloses definite nitrogen retention after urea administration in fifteen of the nineteen cases in which the test was completed. The average net nitrogen retention in the nineteen cases is 2 gm. of nitrogen. Although these studies on humans are entirely inconclusive they do not contradict the results of the present work.

Experimental Methods

Diets were calculated from standard tables, chiefly those of Atwater and Bryant and of the Connecticut Agricultural Experi-

ment Station. Food was weighed and prepared in the diet kitchen of the New Haven Hospital. The subjects refused no food.

Urea solutions were prepared in the laboratory from Merck's urea, which was frequently analyzed by both Kjeldahl and urease methods and always yielded theoretical values. The 20 per cent solution regularly employed for diuretic purposes was used. Through some misunderstanding, in the first three experiments a new assistant entrusted with its preparation, instead of diluting 200 gm. of urea to a liter, made up the solution by adding 1000 cc. of water to 200 gm. of urea. Repeated analyses proved that this method regularly yielded a solution containing 17.4 per cent of urea (8.1 per cent of urea nitrogen). In the last experiment, when the mistake had been discovered, the urea solutions used were analyzed as a double check against their composition.

The subjects usually voided directly into a large flask which was kept in the laboratory refrigerator. In a few instances, however, single specimens were kept in tightly stoppered bottles at room temperature for several hours. The volume of urine was carefully measured each day, the urinals were rinsed with distilled water, and urine and rinsings were made up to a convenient volume for analysis. In all the experiments urine collection was complete.

Stools were collected directly into 2 quart Pyrex glass bean pots which were immediately placed in the laboratory refrigerator. Sulfuric acid was added in generous quantities to prevent loss of ammonia. At the end of each experimental period the combined stools were transferred from the bean pots to a single large bottle. Enough water was added to make a fairly liquid mixture, which was thoroughly shaken. The whole was then passed through a coarse wire sieve. The part which was first retained in the sieve was further washed with water and rubbed through the sieve until only a small amount remained. This was ground in a mortar with a small amount of concentrated sulfuric acid and again strained and washed. This was repeated several times until there remained only a minute amount of coarse fibrous material, apparently cellulose, which was discarded. The homogenous, acidified watery suspension thus obtained was weighed, shaken, and an aliquot taken by weight for analysis. The stools for each period were separated by carmine which was given in capsule form on the morning of the 1st day of each period. By control experiments it

was shown that representative fecal aliquots could be secured by this method, and that nitrogen added to feces in the form of urea could be quantitatively recovered. It was also demonstrated that stools thus treated could be kept for as much as a month without appreciable change in their nitrogen content.

Sweat from D. D. M. (Experiment 2) was analyzed for nitrogen on two occasions. The method used was that of Bost and Borgstrom (7), except that heavy woolen underwear with long sleeves and drawers was substituted for the cotton union suits and stockings of these investigators.

Expired air was analyzed for nitrogen in only one instance, in Experiment 2 on subject D. D. M. A close fitting mouth piece, equipped with a valve, and a nose clip being used, the expired air was passed through strong sulfuric acid for 1 hour. The acid was then analyzed for nitrogen by the Kjeldahl method.

Venous blood was taken for analysis before breakfast at the beginning and end of each period. Nitrogen in the urine, stools, and vomitus was determined by the macro-Kjeldahl method, combined urea and ammonia nitrogen in the urine by the urease method of Van Slyke and Cullen, urine sulfur by Fiske's benzidine titration method. Trichloroacetic or tungstic acid filtrates of blood or serum were analyzed for non-protein nitrogen by the distillation and titration technique of Bock and Benedict. Blood urea was determined by the method of Van Slyke and Cullen. All methods were carefully checked by the usual controls, including frequent blank analyses of reagents and analyses of urea solutions of known strength. Furthermore it was carefully ascertained that urea added to urines and stools could invariably be quantitatively recovered and that the methods used for the collection and preservation of excreta insured against loss of nitrogen.

Finally, when it became apparent that it was impossible during periods of urea administration to recover all the nitrogen administered, specimens were tested by special oxidation methods for nitrogen compounds which could not be split by the usual Kjeldahl technique, and for nitrate nitrogen, with essentially negative results.

Experiments

Experiment 1—The subject of this experiment, D. D. M., was a physician, aged 28, apparently in good health. He received for a

period of 24 days a diet containing 90 gm. of protein and 3000 calories daily. From the 8th to the 14th day inclusive he took

TABLE I

Nitrogen Metabolism of Three Normal Subjects Who Received Urea

Experiment No. and subject	Days	Daily intake		Daily output		Daily N balance	Daily net retention of N*	Body weight	Blood non-protein N	Daily urine urea N	Daily urine non-urea N
		Protein N	Urea N	Urine N	Total N						
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	*mg. per 100 cc.*	*gm.*	*gm.*
1. D. D. M.	7	14.5	0	13.1	14.4	0.1		68.8	23		
	7	14.4	16.2	26.0	27.1	3.5	3.4	68.4	36		
	10	14.4	0	13.1	14.1	0.3	0.2	68.4	42		
								67.9	25		
2. D. D. M.	12	3.3	0	5.3	6.4	−3.1		68.4	30		
	19	3.3	0	3.9	5.0	−1.7		67.1			
	10	3.3	24.3	25.8	26.8	0.8	2.5	65.8	20.†		
	3	3.4	0	8.1	11.0	−7.6	−5.9	64.7	43.†		
								64.6	18.†		
3. C. L. R.	12	3.3	0	5.4	6.3	−3.0		66.2	35		
	16	3.3	0	3.3	4.3	−1.0		63.7			
	7	3.3	24.3	25.2	26.6	1.0	2.0	61.8	16.†		
	6	3.3	0	4.1	5.4	−2.1	−1.1	61.1	27.†		
	3	13.1	0	5.1	6.2	6.9	7.9	61.2			
								61.0	27.†		
4. P. H. L.	5	14.5	0	13.5	14.7	−0.3		77.6	19.†	11.5	2.0
	9	14.4	16.2	27.5	28.5	2.1	2.5	77.3	23.†	25.3	2.2
	9	14.4	24.3	35.3	36.3	2.4	2.7	75.8	26.†	33.2	2.1
	9	14.4	16.2	28.1	29.0	1.7	1.9	75.1	23.†	26.0	2.2
	3	14.4	0	12.8	14.1	0.2	0.5	75.1	26.†	10.8	2.0
								75.0	25.†		

* Calculated as the difference between the nitrogen balance of the fore period and subsequent periods.

† For non-protein nitrogen marked *s*, serum was used instead of blood.

each day, in addition, 34.7 gm. of urea (16.2 gm. of urea nitrogen). The urine and stools were analyzed for nitrogen. The data, calculated as were those of the previous experiments, are given

in Table I. The daily nitrogen metabolism is graphically represented in Fig. 1.

The diet was chosen to approximate as nearly as possible the usual diet of the subject, in order that nitrogen equilibrium might be attained as rapidly as possible. The effort appears to have been successful. The daily nitrogen excretion of the 1st week varied within the narrow limits, 12.7 to 15.4 gm., and the nitrogen output for the whole week exactly equalled the intake. During the urea
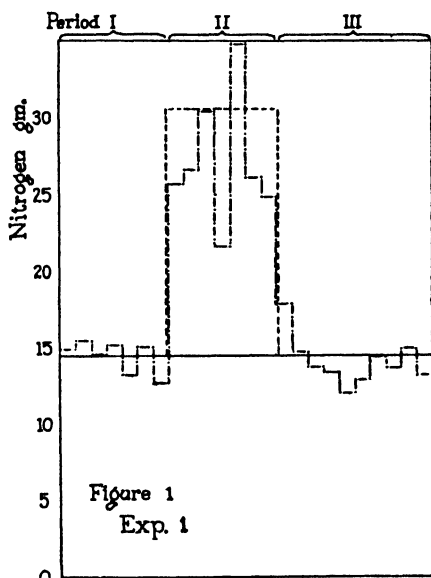
FIG. 1. Nitrogen metabolism of normal subject receiving urea (Experiment 1). The solid line indicates protein nitrogen intake; broken line, added urea nitrogen; dot and dash line, total nitrogen excretion in urine and feces.

period there was an average daily retention of 3.4 gm. of nitrogen. Moreover, the amount retained during the last days of the period was quite as great as that at the beginning. (The large daily variations of nitrogen output shown in Fig. 1 during Period II are due to the fact that the urea was not always taken at the same time of day.) In the after period little or none of the retained nitrogen was swept out. The balance for the whole 10 day after period indicates exact nitrogen equilibrium.

Experiments 2 and 3—Two normal males, D. D. M. of Experiment 1, and C. L. R., a medical student, 26 years old, in good health except for a slight chronic bronchiolitis, were the subjects of these
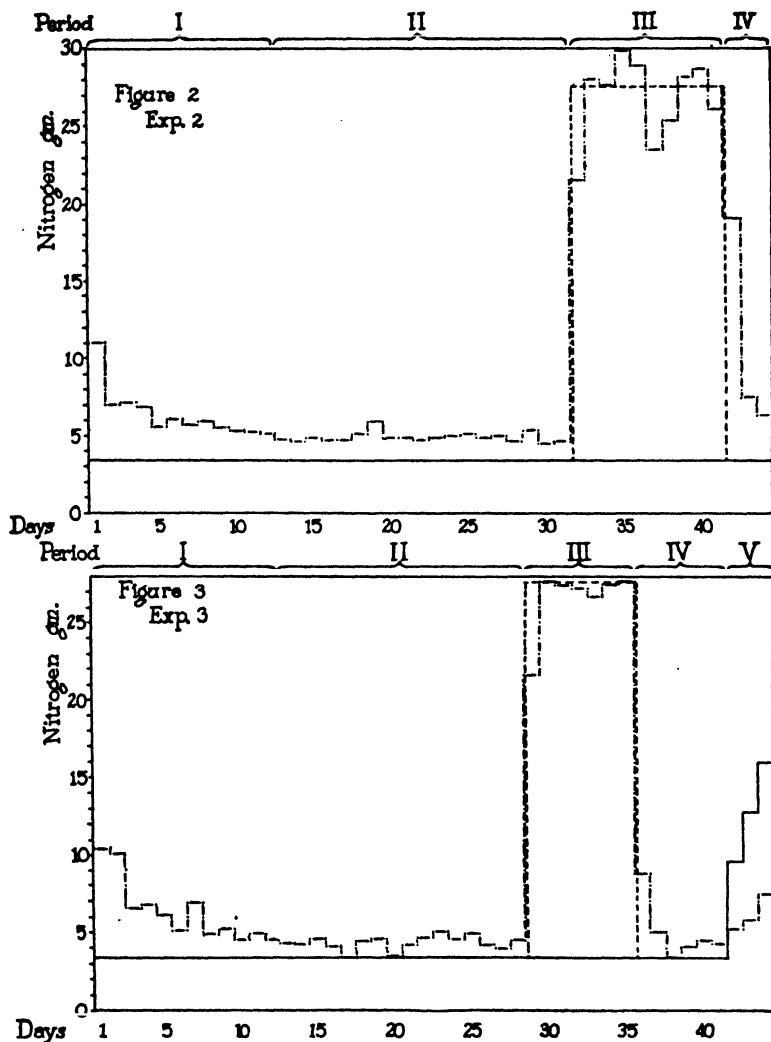
FIGS. 2 AND 3. Nitrogen metabolism of normal subjects receiving urea in addition to a low protein diet (Experiments 2 and 3). The solid line indicates protein nitrogen intake; broken line, added urea nitrogen; dot and dash line, total nitrogen excretion in urine and feces.

experiments.   They received, for preliminary periods of 28 and 31 days respectively, diets containing only 20 gm. of protein daily. That of D. D. M. contained 3000 calories, that of C. L. R. who was naturally better nourished, only 2500.   At the end of the preliminary period 52.1 gm. of urea (24.3 gm. of urea nitrogen) were taken daily in addition to the original diets.   The urea was continued for 10 days in the case of D. D. M., but for only a week in the case of C. L. R.   At the close of these periods D. D. M. continued on the original diet for 3 days, C. L. R. for 6 days.   C. L. R. was then given increasing amounts of protein for 3 days.   In these experiments the urine was analyzed for total sulfur as well as total nitrogen.   Basal metabolism determinations were also made at frequent intervals.   The results of the experiments are summarized in Table I and the daily nitrogen exchange is illustrated in Figs. 2 and 3.

It was intended in these experiments to add information on several points.   (1) Would the retention of urea nitrogen be greatly accelerated after a certain wastage of body protein had occurred and nitrogen metabolism had been reduced to a low and relatively constant level?   (2) Was the retention associated with changes in sulfur excretion which would be interpreted as indications of protein synthesis?   (3) Would the basal metabolism, after it had been reduced by protein deprivation, be restored to the normal level by urea feeding?

But few of these aims were realized.   The only certain result of the experiment is complete confirmation of previous studies.   In the urea periods part of the administered nitrogen again remained unrecovered.   The quantities unaccounted for were no larger, however, but rather smaller than those lost in other experiments. In the 1st day of each period a small amount of the unrecovered nitrogen was swept out; after that the nitrogen excretion fell to its original low level.

Experiment 2 has been divided into five periods, Experiment 3 into six, the last representing the 3 days of increased protein feeding.   In both, during the initial periods the nitrogen excretion gradually fell to a minimum, which was maintained at a relatively constant level throughout the second period.   The second periods are used for the estimation of basal nitrogen excretion to compare with that of subsequent periods.

The sulfur figures were inconclusive.   In Experiment 2 urinary

sulfur remained constant during Periods II and III, while in Experiment 3 it fell during the urea period.   Both subjects excreted less sulfur in the recovery periods than they did in earlier periods. Basal metabolism data are not reported.   During the urea period both subjects developed acute upper respiratory infections which made the respiratory studies difficult and unreliable.
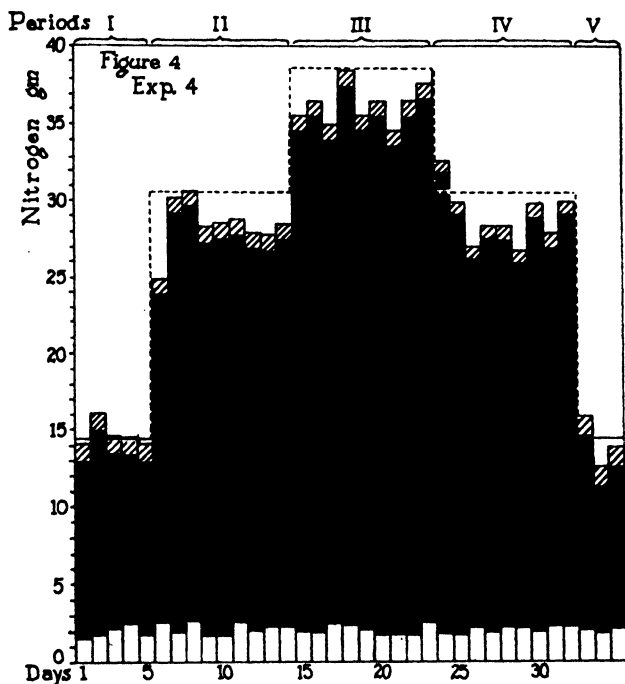
FIG. 4. Nitrogen metabolism of normal subject receiving urea (Experiment 4).   The horizontal solid line represents protein nitrogen intake, the broken line the added urea nitrogen.   The unshaded areas represent non-urea nitrogen; solid areas, urea nitrogen of the urine; the diagonally lined areas indicate fecal nitrogen.

The last period of Experiment 3 demonstrates that the individual who has been starved of protein will store large amounts when given an adequate supply.   Furthermore, protein nitrogen fails of recovery in far larger proportions than urea nitrogen. During the urea period, with an intake of 27.6 gm. of nitrogen, the unrecovered nitrogen was only 2.0 gm. per day.   During the pro-

tein period a net daily disappearance of 7.9 gm. of nitrogen was observed when only 13.1 gm. were given.

Experiment 4 resembles in most respects Experiment 1. The subject, P. H. L., a medical student, apparently in good health, received for 35 days a diet containing 90 gm. of protein and about 3000 calories daily. After 5 days in which nitrogen equilibrium was established he was given daily for three successive periods of 9 days each 34.7, 52.1, and 34.7 gm. of urea (16.2, 24.3, and 16.2 gm. of urea nitrogen) respectively. The urinary urea as well as the total nitrogen excretion was determined. The results of the experiment are summarized in Table I and the daily nitrogen metabolism is graphically represented in Fig. 4.

Again, as in previous experiments, there is the same unaccountable failure to recover, during the urea periods, all the nitrogen ingested. The quantity lost is of the same order of magnitude as that in the other experiments on normal subjects and is not significantly greater when 52.1 gm. of urea are given (Period III) than when 34.7 gm. are given (Period II). There is still a definite daily disappearance at the end of 27 days of urea feeding, and, although the daily loss is somewhat smaller in the last urea period than in the first, the diminution is not sufficiently marked to permit one to conclude that the capacity to retain nitrogen, if the nitrogen is retained, is becoming exhausted. During the after period of 3 days nitrogen equilibrium was established almost at once; the unrecovered nitrogen of the urea periods was not swept out. Again perfect nitrogen equilibrium appears to have been attained in Periods I and V.

The non-urea fraction of the urinary nitrogen remains remarkably constant through the experiment. This indicates that the nitrogen discrepancy involves urea only.

Analysis of sweat on two occasions in Experiment 2, during extremely hot weather, yielded a total nitrogen excretion of 0.3 gm. in one 24 hours of the preliminary period, and 0.1 gm. in 24 hours of the urea period. In the same experiment expired air collected for 1 hour immediately after a dose of 60 gm. of urea was found to contain only 0.027 gm. of nitrogen, an amount which, by the technique employed, is negligible. Moreover, as the subject had just taken the urea and was somewhat salivated when the collection of expired air was begun, the amount recovered is prob-

ably far greater than it would have been at other times during the same 24 hours.

It appears from these experiments that when urea was fed to human subjects a substantial portion of the administered nitrogen was not recovered in the urine and feces.   The constancy of the fecal nitrogen throughout the course of each experiment confirms previous observations that ingested urea is completely absorbed from the alimentary tract.   Excretion in the sweat has not been certainly excluded.   Collection and analysis of sweat presents certain inherent difficulties.   It seems highly unlikely, but not impossible, that even the relatively crude technique employed in the sweat experiments could have recovered only one twenty-fifth of the daily excretion, 0.1 gm. instead of the "net retention" of 2.5 gm.   Expired air as a channel for nitrogen excretion seems to be more definitely excluded.   That the failure to recover the nitrogen is due neither to technical errors nor to the conversion of this nitrogen into some unusual form appears to be demonstrated by the control experiments.

The constancy of the non-urea nitrogen excretion in Experiment 4 indicates that it is urea nitrogen which is lost.   If urea is freely diffusible and evenly distributed throughout the fluids of the body, the retention of such quantities should affect the blood non-protein nitrogen.   The experimental data, however, agree with those of other observers in showing that the administration of as much as 52 gm. of urea to a normal individual in the course of the day does not appreciably influence the blood non-protein nitrogen taken the following morning.   Table I gives but a slight idea of the actual magnitude of the nitrogen retentions that were observed, because it presents only the daily values.   In Table II are presented the total quantities actually lost in the urea periods.   In Experiment 4 at the end of 27 days, 63 gm. of nitrogen were unrecovered.   If this had been retained as urea and distributed evenly through 70 per cent of the body mass. it would have increased the serum non-protein nitrogen by 120 mg. per 100 cc.   In actual point of fact the serum non-protein nitrogen rose only from 23 to 26 mg. per 100 cc.

The most obvious objection to the data lies in the failure to

analyze diets, a procedure which was hardly feasible in experiments which extended over such long periods.   Undoubtedly there is a variable error in the estimation of diets from standard tables. However, it is difficult, if not mathematically impossible, to conceive of any fortuitous conjunction of circumstances which could have caused constant errors in diet composition to occur during,

TABLE II

Total Nitrogen Storage of Normal Subjects Who Received Urea

Urea periods are indicated in bold face type.

Experiment No. and subject	Days	Total N			
		Intake	Excretion	Balance	Net retention
		gm.	*gm.*	*gm.*	*gm.*
1. D. D. M.	7	101.5	100.8	0.7	
	7	**214.2**	**189.7**	**24.5**	**23.8**
	10	144.0	141.0	3.0	2.0
2. D. D. M.	12	39.6	76.8	−37.2	
	19	62.7	95.0	−32.3	
	10	**276.0**	**268.0**	**8.0**	**25.0**
	3	10.2	33.0	−22.8	−17.7
3. C. L. R.	12	39.6	75.6	−36.0	
	16	52.8	68.8	−16.0	
	7	**193.2**	**186.2**	**7.0**	**14.0**
	8	19.8	32.4	−12.6	−6.6
	3	39.3	18.6	20.7	
4. P. H. L.	5	72.5	73.5	−1.0	
	9	**276.3**	**256.5**	**19.8**	**21.6**
	9	**348.3**	**326.7**	**21.6**	**24.3**
	9	**275.4**	**261.0**	**14.4**	**17.1**
	3	43.2	42.3	0.9	1 5

and only during, the urea periods.   If the attainment of nitrogen equilibrium in Experiments 1 and 4 be discounted as accidental, the absolutely constant difference between these periods and the urea periods still requires explanation.   In Experiments 2 and 3, if diet errors are to explain the findings one must conclude that during the whole of the urea period two normally nourished subjects, receiving low protein diets, maintained nitrogen equilibrium, while actively working, on less than 20 gm. of protein a day.

The experiments indicate only that when large doses of urea are given to human beings daily for a period of 7 days or longer, a significant fraction of the urea nitrogen cannot be recovered in either urine or feces by the Kjeldahl procedure or its usual modifications. Concerning the fate of the lost nitrogen they yield no information. That it is retained for any useful purpose, either as protein or urea, seems unlikely. That it may be excreted by the skin or in some unusual form is not entirely precluded by the control experiments.

SUMMARY

Urea was fed in doses of 35 to 52 gm. daily in four experiments on three normal subjects. The nitrogen metabolism and blood non-protein nitrogen were determined for periods before, during, and after the administration of urea under various circumstances.

During the administration of urea, in every case, a considerable amount of nitrogen could not be recovered in either urine or feces.

This apparent daily retention continued as long as urea was given.

It was not attended by a comparable increase of blood non-protein nitrogen.

The nitrogen not recovered during periods of urea administration was not swept out in subsequent periods.

Analysis of sweat and expired air did not reveal unusually large quantities of nitrogen during urea administration.

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CONCERNING THE USE OF COTTONSEED MEAL IN THE DIET OF THE RAT*

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Many experiments which have been carried out on laboratory animals in recent years to answer questions pertaining to the use of cottonseed products in the diet of domestic animals have led to controversies over the toxicity of cottonseed meal to the rat. Cottonseeds which contain approximately 19 per cent oil and 20 per cent protein are seldom used to supplement the proteins of a diet because of their content of a toxic substance called gossypol. Cottonseed meal which contains twice as much protein and much less physiologically active gossypol is used extensively for this purpose, and cottonseed flour and cottonseed protein have been suggested as possible foods for man. Since the rat is commonly used for the determination of the adequacy and fitness of certain foods in the diet of man and lower animals, agreement as to its dietary habits and response to certain alterations of the diet is important.

That cottonseed meal is toxic to rabbits and guinea pigs seems to be an established fact. That the meal is toxic to rats is less well established since these animals do not respond so readily to cottonseed meal diets but may display signs of injury only during a late period of life or just following the period of most rapid growth. In such cases, diagnosis of an abnormal condition as being typical cottonseed meal injury is of doubtful significance unless based upon an examination of growth rates together with the general health and condition of the animals as compared to those of con-

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trol animals receiving some other form of protein or cottonseed meal in which the gossypol has been removed or destroyed. Even then other factors may have contributed to the symptoms displayed. Obviously, functional disturbances other than those due to gossypol, *per se*, may become more pronounced during the later period of growth and in some instances gossypol or cottonseed meal injury becomes complicated with nutritional deficiency disorders. Investigators have referred to the unhealthy condition displayed by animals which have been fed for long periods upon heavy cottonseed product diets as chronic injury in contrast to an acute injury which so often proves fatal early in the life of the animal.

Dowell and Menaul (1) working in this laboratory were the first to show that cottonseed meal of proven toxicity to swine could be rendered non-toxic by autoclaving it at 15 pounds pressure for 20 minutes. Their results and the results of one later experiment published by the author (2) have been referred to recently by Clark (3) in this manner: "in spite of the claims made, the growth rate of rats fed on the autoclaved cottonseed meal is inferior to that attained by the rats fed upon the unautoclaved meal." Dowell and Menaul made no claims as to the effect of feeding cottonseed meal to rats. Their results with swine are quite conclusive and have been confirmed. The author presented the gains in weight made by swine and a limited number of rats during a short feeding period on diets containing autoclaved, steamed, and untreated cotton seeds and cottonseed meal. The results agreed with what might be inferred from earlier work on cotton seeds by Osborne and Mendel (4), and seemed to justify the general conclusion that, "Cooking the meal increases the amount which may be safely fed over a period of time." However, in the same article and later ones (5), attention was called to the fact that rats are not so susceptible to the effects of cottonseed meal in their diet as are some other animals, that they not only show great individual variations in this respect, but that their response to such feeding is modified by variations in the gossypol content of different samples of meal. Doubtless the cottonseed meal used in the study referred to was unusually toxic.

Effect of Supplements[1]

The growth rate and well being of animals being fed upon cottonseed meal will vary greatly with the supplements in the diet. This has been brought out in practical feeding trials and in Clark's work in which supplements were supplied in the form of casein and a salt mixture containing ferric citrate. The extent to which iron salts may offset the effects of gossypol is apparent from results previously reported (6). Other supplements may have specific effects upon gossypol, or by their inherent nature have properties which prevent the occurrence of certain physiological disturbances attributed to an overfeeding of cottonseed meal.

EXPERIMENTAL

The experiments herein reported were planned to establish evidence for the contention that cottonseed meal contains physiologically active gossypol in amounts sufficient to influence the growth and well being of rats. Such evidence was to be obtained from the results of controlled feeding experiments in which advantage was taken of previously acquired information that iron salts are efficacious against gossypol injury and that gossypol is destroyed when heated to high temperatures in the autoclave. The results substantiate the viewpoint that prolonged feeding of cottonseed meal is injurious to rats; that the injury produced is due mainly to gossypol and its compounds, but that it may not become apparent during a short feeding period; that the injury is delayed for some time by supplementing the diet with iron salts; and finally, that the toxicity of the meal is reduced by autoclaving.

As in previous studies, the diets were composed principally of natural food materials which together with the cottonseed meal were believed to supply sufficient amounts of the necessary dietary essentials.

The basic diet containing cottonseed meal to which different amounts of iron salt were added, contained the following ingredients.

Cottonseed meal (standard or autoclaved).................. 40
Yellow corn... 40

---

[1] A complete discussion of the effect of supplements in cottonseed meal rations is to be made later in a report from this Station.

The autoclaved meal was prepared as previously described (2). The standard meal, which refers to cottonseed meal as sold on the market, was a composite sample containing 0.01 to 0.02 per cent gossypol and 0.70 per cent d-gossypol. The gossypol was determined by the method of Schwartze and Alsberg (7) and the d-gossypol, which refers to the form that is not removed from the meal by extraction with ether, was determined by Sherwood's method (8). The amount of iron used in the diets in the form of $FeSO_4 \cdot 7H_2O$ was far in excess of that which would be recommended to make up for an iron deficiency and was added solely for its antidotal properties. Previous studies (6) had shown a roughly quantitative relationship between the amount of gossypol in the diet and the amount of iron required to offset its deleterious effects.

The rats taken from our regular stock colony were kept in individual cages during part of the experiment to allow for accurate records of the food consumption. At other times the males and females on similar diets were allowed to breed in order to furnish reproduction records. The usual precautions necessary in conducting experiments of this kind were observed, with the exception of room temperature control. During the latter part of the experiment extremely hot weather prevailed.

DISCUSSION AND RESULTS

The gains in weight made during four successive 30 day periods by rats fed upon the basic diets with and without the addition of different amounts of iron are presented in Table I. The data are presented in this manner in preference to graphic representation to facilitate a comparison of the individual gains made by rats fed upon autoclaved and unautoclaved meal. It is to be noted that during the first 60 days all animals made about normal gains.

Table I shows that during the last 60 days of the experiment the animals receiving the standard meal in their diets without the iron additions (Group A) almost invariably made smaller gains than their litter mates receiving the autoclaved meal (Group B). At

TABLE I

Rats on Cottonseed Meal Diets Supplemented with Iron

Rat	Initial weight	Gain made during successive 30 day intervals					Final weight	Remarks

Group A, standard meal

Rat	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	Remarks
100 a ♂	60	88	34	35	11		228	Young of Rat 100 c died 1 wk.
100 b ♂	60	80	22	28	− 7		183	after birth. Average daily
100 c ♀	50	52	24	24	−15		135	food intake 14 gm.
100 d ♀	50	58	25	17	− 5		145	

Group B, autoclaved meal

101 a ♂	60	90	50	32	28		260	7 young born to Rat 101 c,
101 b ♂	65	95	50	30	20		260	6 born to Rat 101 d, all
101 c ♀	50	75	25	10	10		170	lived. Average daily
101 d ♀	45	55	32	18	10		160	food intake 16 gm.

Group C, standard meal + 0.25 per cent iron salt

105 a ♂	65	75	95	−10	−20		205	Animals weighed approxi-
105 b ♂	65	80	75	−10	−45		165	mately 220 gm. after 60
105 c ♂	55	85	75	10	−31		194	days on experimental diet.
105 d ♂	55	70	95	− 5	−20		195	Average daily food intake 15 gm.

Group D, standard meal + 0.5 per cent iron salt

102 a ♂	50	85	80	55	10		280	7 young born to Rat 102 c;
102 b ♂	70	85	90	50	10		305	2 died. 7 young born to
102 c ♀	59	58	58	20			195	Rat 102 d; 1 died. Aver-
102 d ♀	45	60	45	10	10		170	age daily food intake 18 gm.

Group E, standard meal + 1 per cent iron salt

103 a ♂	60	85	73	42	10		270	5 young born to Rat 103 f,
103 b ♂	70	82	73	−15	5		215	5 born to Rat 103 g, 6
103 c ♂	70	85	65	− 5	− 5		210	born to Rat 103 h, all lived.
103 d ♂	70	75	80	−35	15		205	Average daily food intake
103 e ♂	70	86	89	− 5	− 5		235	17.5 gm.
103 f ♀	60	57	73	−20	10		180	
103 g ♀	65	52	48	10	5		180	
103 h ♀	50	60	40	10	7		167	

TABLE I—*Concluded*

Rat	Initial weight	Gain made during succes- sive 30 day intervals					Final weight	Remarks
Group F, standard meal + 1.5 per cent iron salt								
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		
106 a ♂	65	90	75	10	7	247		
106 b ♂	70	70	50	45	15	250		
106 c ♂	60	85	40	20	15	220		
106 d ♂	60	95	25	35	5	220		
Group G, standard meal + 2 per cent iron salt								
104 a ♂	55	75	80	45	20	275	9 young born to Rat 104 d.
104 b ♂	50	83	82	55	15	285	Average daily food intake
104 c ♂	55	70	65	60	15	265	20 gm.
104 d ♀	58	37	50	25	− 5	165	
Group H, autoclaved meal + 0.1 per cent iron salt								
109 a ♂	65	100	55			220	Experiment closed at end
109 b ♂	65	90	55			210	of 60 days
109 c ♀	48	52	30			130	
109 d ♀	60	72	28			160	
Group I, autoclaved meal + 1 per cent iron salt								
107 a ♂	65	100	45	5	20	235	
107 b ♂	65	105	45	10	45	270	
107 c ♂	65	120	45	5	40	275	
107 d ♂	65	115	42	26	7	255	

the close of the experiment, the unhealthy condition associated with roughness of coat and the uncleanliness of Group A was very noticeable. At times they became extremely nervous or excited and appeared to breathe unusually rapidly. This was often followed by a period of depression during which time the animals became listless and did not offer resistance to handling and being moved about. The female rats appeared to be more susceptible to injury than the males and this was especially noticeable after the former had given birth to a litter of young. During the lactation period and following it, the females in Group A did not make consistent gains with a return to normal weight. Furthermore, the young did not appear thrifty and vigorous and at weaning time usually weighed less than the young born to females in

Group B. Results similar to these have been previously encountered (5).

In contrast to the above results is the growth made by rats which received diets of the standard meal supplemented with 0.5 to 1.0 per cent $FeSO_4 \cdot 7H_2O$. Smaller amounts of iron proved to be less effective, whereas greater amounts as were present in the diets of the animals in Groups F and G did not appear to stimulate growth in the same proportion, nor did these large amounts appear to be detrimental to the animals' health. Food consumption was noticeably increased as a result of the iron additions. Because of the variations observed in the growth and condition of the rats which received 1 per cent iron salt, conclusive statements as to the optimal ratio of meal to iron cannot be made, but it appears to be approximately 100:1, calculated as $FeSO_4 \cdot 7H_2O$. Addition of iron to the basic diet containing autoclaved meal did not result in better growth.

These observations when considered in their relation to those which have been made upon other animals fed upon similar diets indicate the extent to which gossypol is a determining factor in the judicious use of cottonseed meal as a food. The autoclaving process seems to be the final step necessary in changing the gossypol as it exists in an extremely toxic condition in the seeds to a less and possibly non-toxic one in the final product. The effectiveness of this process has been discussed elsewhere (9).

No explanation is offered at this time for the results reported by Clark (3) in which the standard meal proved to be superior to the autoclaved meal for the growth of rats. Perhaps the diversity of opinion concerning the merits of the autoclaved meal has arisen from an incorrect interpretation of results obtained with different supplements in the diet or possibly from conclusions drawn from feeding experiments of too short duration.

The opinion that rats show variations in their susceptibility to gossypol is confirmed by the figures presented in Table I, and strengthened by the results of the following experiment. Six rats were injected intraperitoneally with 25 mg. of gossypol per kilo of body weight. Three of the rats died the following day and of the three remaining, one became greatly distended in the abdominal region 3 days later and was killed, whereas the other two gained between 2 and 3 gm. per day during the next 15 days. When 1½

times as much gossypol was similarly injected into six other rats, four died the following day and the remaining two gained between 2 and 3 gm. per day during the following 15 days.

SUMMARY

The unhealthy condition and retarded growth of rats fed for 120 days upon diets which contained 40 per cent cottonseed meal were shown to be due to the presence in the meal of appreciable amounts of physiologically active gossypol. The effects of the gossypol became apparent during the last 60 days of the experiment.

Improvement in the growth and well being of animals fed upon cottonseed meal diets was brought about in two ways: (1) by administering inorganic iron ($FeSO_4 \cdot 7H_2O$) which is efficacious against gossypol injury, and (2) by replacing the ordinary meal in the diet with an autoclaved product which contains little if any active gossypol.

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THE NATURE OF THE PROTEINS AND LIPIDS SYNTHE-SIZED BY THE COLON BACILLUS*

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In recent years it has been frequently suggested that the bacteria commonly present in the gastrointestinal tract of animals may, because of their synthetic powers, play a rôle in nutrition. Some investigators are of the opinion that the retention of nitrogen by animals on diets devoid of protein can be ascribed to bacterial action, and others believe that the results obtained in investigations devised to prove the synthesis of certain lipids by man are entirely due to bacterial synthesis. While it is generally accepted that bacteria can synthesize certain lipids, both the commonly occurring fatty acids, stearic, palmitic, and oleic, and some of the phospholipids, their power is somewhat limited, since they do not seem to possess the ability of building up the sterols. The total lipids in the dry organisms usually vary from 5 to 10 per cent. Occasionally, as in the case of the tubercle bacilli, the fat content of the dry organisms is found to be as high as 40 per cent, but even under these circumstances no sterols or fatty acids more highly unsaturated than linoleic acid have been demonstrated as being present. The total nitrogen content of most desiccated bacteria amounts to about 10 per cent. While there is evidence recorded in the literature pointing to the synthesis of many of the naturally occurring amino acids by these microorganisms, many of the data presented are open to criticism, since the organisms studied were usually cultured on media containing nitrogenous substances at least as complex as the peptones.

Of the organisms which are present in the gastrointestinal tract of man and which therefore may play a rôle in nutrition, the colon

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bacillus is a representative one. Our knowledge concerning the products that can be synthesized by this organism from simple precursors is not very definite. Perhaps the most detailed study of this question was made in the laboratory of Dr. Vaughan more than two decades ago by Leach (1) who grew large amounts of the organism on a peptone medium. The lipids contained in these organisms were removed by extraction, but unfortunately no report was made concerning their nature. The proteins were studied, but most of the work concerning their amino acid content is open to criticism since the material fractionated by means of the Kossel and Kutscher procedure was an incomplete hydrolysis mixture of the bacterial proteins. The presence of lysine was, however, definitely established.

Since data are lacking concerning the products which can be synthesized by the colon bacillus from simple known precursors, the investigation to be reported herein seemed desirable. A pure strain of the organism (*Bacillus coli*, Jordan) was obtained and cultured on media containing inorganic salts, carbohydrate, and nitrogenous compounds of known composition. These media are described in Table I. It is evident, from the data in Table I that the media are "fat"-free, contain the same amounts of water, agar, inorganic salts, and glucose, but differ with respect to their sources of nitrogen. In one, the nitrogen was supplied in the form of *d*-alanine, whereas, in the other, the nitrogen was present as *l*-cystine (prepared from hair). The organisms were cultured in air in Roux flasks for 48 hours, and the growths obtained washed off the media with physiological saline and separated by centrifugation. The solid material thus obtained was macerated with cold saline and the resulting suspensions centrifuged. Biuret tests made on all of the supernatant liquids were invariably negative. These fluids were therefore discarded. The moist organisms were transferred to dark colored bottles with 95 per cent alcohol. At the end of 48 hours the solid material was filtered off, the extracts being retained, and the organisms washed back into the original bottles with a large volume of absolute alcohol. They were then subjected to extraction with the cold solvent for a period of 1 week. The solids were again filtered off, the extract being combined with the one previously obtained with 95 per cent alcohol, and the organisms returned to the bottles with absolute alcohol

with which they were then extracted for another 1 week period. This process with absolute alcohol was repeated once more, making a total of three extractions with this particular solvent. The bacteria were then extracted for a 48 hour period with cold anhydrous ethyl ether and for a similar length of time with cold chloroform. They were finally transferred to a Buchner funnel and thoroughly washed with cold anhydrous ethyl ether. The washings and all of the extracts were combined, concentrated under reduced pressure, and the resulting concentrates extracted with cold petroleum ether (b.p. 20–40°). This petroleum ether extract was made up to a definite volume from which aliquots

TABLE I

Composition of Media

	Medium A	Medium B
	cc.	*cc.*
Water..	7000	7000
	gm.	*gm.*
K_2HPO_4......................................	7	7
KH_2PO_4......................................	7	7
$CaCl_2$..	0.7	0.7
$MgSO_4$..	1.4	1.4
Glucose......................................	10	10
Agar...	140	140
Alanine......................................	7	None
Cystine......................................	None	2.67

were taken for analyzing the lipids synthesized. The "defatted" organisms were dried to constant weight *in vacuo* at room temperature.

In Table II are grouped some of the results obtained. It is apparent from that summation that so far as the total nitrogen, total solids, and ash contents are concerned little difference exists between the organisms cultured on the two media. There is, however, considerable difference between the amounts of lipids synthesized; the organisms grown on the alanine medium contained more than twice the amounts of fatty substances than those cultured on the other medium. In Table III more detailed information is given concerning the nature of these lipids. The organisms obtained from the cystine medium contained only 3.6

per cent of total lipids, whereas the other ones had 7.8 per cent of the substances. More than one-sixth of the total lipids removed from the organisms grown on Medium A (alanine) were, as calculated from their phosphorus content, found to be in the form of phospholipids, whereas only traces of such lipids could be detected in the organisms obtained from Medium B. No data

TABLE II

Analysis of Bacillus coli Cultured on Alanine and Cystine Media

	Organisms cultured on	
	Alanine medium	Cystine medium
Moist organisms, *gm*................................	242	56
Dry defatted organisms, *gm*.....................	28.2	5.8
Total lipid as per cent of dry bacteria..........	7.8	3.6
Ash as per cent of dry bacteria.................	5.1	4.6
Total N in defatted bacteria, *per cent*..........	10.28	9.96

TABLE III

Nature of Lipids Synthesized by Colon Bacilli from Lipid-Free Precursors

	From alanine medium	From cystine medium
Total lipids in dry organisms, *per cent*..........	7.8	3.6
Iodine No.		
Total lipids...................................	25.6	31.0
" fatty acids...........................	37.4	39.2
Total fatty acids in total lipids, *per cent*........	70	79
Saponification No..............................	214	Not determined
Non-saponifiable matter in total lipids, *per cent*..	6.4	5.7
Phospholipids in total lipids, *per cent*..........	17.4	Trace
Cholesterol*..................................	None	None

    * As determined by the Liebermann-Burchard and Salkowski tests.

are available to explain these marked differences and further work is necessary as a result of these findings. In this connection it may be well, at this point, to note that the growths obtained on the alanine medium were invariably much larger than those secured by culturing the organism in the other manner. During the incubation period on the cystine medium considerable hydro-

gen sulfide seemed to be evolved. The presence of the gas became evident as soon as one entered the room in which the bacteria were being cultured. Proof of the presence of the gas was obtained by suspending moist lead acetate paper into the flasks containing the bacilli. No indication of the presence of this gas was obtained in tests made on the other cultures. Cystine is considered to be a toxic substance if introduced in sufficiently large amounts into animal organisms and hydrogen sulfide is known to be a very poisonous gas. It is possible that some relationship might exist between the presence of these toxic substances and the poor growths and low phospholipid contents referred to above. In spite of these marked differences, the lipids removed from the organisms obtained from Media A and B resemble each other in that the iodine numbers of the "fats" secured from both sources are very similar, varying only from 25.6 to 31. These values are like those recently reported by Terroine, Hatterer, and Roehrig (2) who found the iodine number of the total lipids of an organism (bacille de la Fléole) to be 30. Anderson and Chargaff's (3) figure for the liquid fatty acids of the tubercle bacillus was low. Of the unsaturated fatty acids, oleic is perhaps the only one present in the lipids isolated from the colon bacilli. The occurrence of the more highly unsaturated ones is very doubtful, inasmuch as no precipitation took place when bromine was added to the cold petroleum ether solution of the total fatty acids. In this respect these bacterial lipids differ from most naturally occurring fats. Unlike these other "fats" the lipids in *Bacillus coli* contain none of the sterols that can be detected by means of the Liebermann-Burchard or Salkowski tests. These findings are in line with the very recent ones of Miyoski (4) who made chloroform extracts of as much as 10 gm. of dry colon bacilli as well as of other bacteria and failed to demonstrate measurable amounts of cholesterol by means of either the digitonin method or color tests. Of the lipids that have been removed from bacteria none seems to contain the widely distributed sterols. In this respect bacteria differ from many molds and yeasts for such fungi contain these solid alcohols.

From the preceding data it is apparent that the lipids synthesized under the conditions of this investigation can be characterized by their low iodine number, by the absence of fatty

acids more highly unsaturated than oleic, and by their failure to contain sterols, detectable by means of the common color reactions for cholesterol. It is conceivable that different products might be synthesized by these organisms in the gastrointestinal tract, since the conditions existing therein are quite different from those obtaining in our experimental procedure. Some information regarding this question is found in a paper by Sperry (5). This investigator studied the lipids that had been extracted from bacteria obtained from dog feces. Making use of the well known lead salt method, Sperry separated the solid from the liquid fatty acids and found that the latter predominated, the mean ratio of liquid to solid acids being 1.77:1. This suggests that the iodine number of the mixed fatty acids might be higher than those reported by us, but such a conclusion is not permissible until more work has been done on such lipids, especially since Anderson and Chargaff (6) have definitely shown that the liquid fatty acid fraction of fatty acids obtained from the tubercle bacillus contains considerable amounts of saturated fatty acids that are liquid and form ether-soluble lead salts. Whether similar products are present in the lipids of the organisms commonly present in the gut is unknown, but until such information is obtained no definite conclusion can be arrived at by comparing our data with those of Sperry.

The material from which the lipids had been extracted was used to obtain information regarding the nature of the proteins synthesized. Some of the data concerning this question are grouped in Table IV, where the distribution of the various forms of nitrogen as determined by the Van Slyke partition method are given. Additional data dealing with the general problem are shown in Table V in which the values for tyrosine and tryptophane obtained by means of the Folin and Ciocalteu (7) method are recorded. From the data grouped in Tables IV and V it appears as if, with the possible exception of their lysine content, little difference exists between the proteins analyzed. These proteins might be characterized by their high content of the bases and their lack of cystine. The results in Table IV may, however, be inexact, since the substances analyzed were not pure protein and might therefore contain products that would cause erroneous results in the determination of the bases. Furthermore, small amounts of

cystine might not be detected as a result of the procedure used, inasmuch as some cystine is usually destroyed during the long hydrolysis necessary in the method and likewise since cystine phosphotungstate is somewhat soluble. For these reasons other methods were employed to determine these acids. The method of Vickery and Leavenworth (8) as modified by Calvery (9) for small

TABLE IV

Distribution of Nitrogen in Proteins of Bacillus coli as Determined by the Van Slyke Distribution Method

Results are expressed as per cent of total nitrogen.

	Organisms cultured on	
	Alanine medium	Cystine medium
	per cent	*per cent*
Amide N...............................	11.30	10.80
Acid-insoluble melanin N....................	2.39	2.64
Alkali-insoluble melanin N..................	2.41	3.10
Arginine N..............................	15.40	16.30
Histidine N.............................	10.61	10.41
Lysine N................................	12.42	8.90
Cystine N...............................	None	None
Amino N in filtrate......................	42.90	45.71
Non-amino N in filtrate..................	3.20	3.41

TABLE V

Tyrosine and Tryptophane Content of the Proteins of Bacillus coli

	Tyrosine N, per cent of total N	Tyrosine, per cent of protein	Tryptophane N, per cent of total N	Tryptophane, per cent of protein
From alanine medium............	0.90	1.21	1.57	1.18
" cystine "	0.84	1.08	1.48	1.07

amounts of protein was used to determine the bases, and the procedure of Folin and Marenzi (10) for cystine. Due to lack of material, it was impossible to determine the bases in the proteins obtained from the organisms cultured on the cystine medium. The results for the bases obtained by this isolation method were, as might be expected, lower than those secured with the Van Slyke method. Thus the percentage of arginine nitrogen as calculated

from the amount of arginine flavianate was 13.08 per cent, the histidine nitrogen as determined from the weight of histidine flavianate 6.51 per cent, and the lysine nitrogen as calculated from the amount of lysine picrate 5.7 per cent. These data furnish positive evidence for the synthesis of the bases by the colon bacillus from so simple a precursor as *d*-alanine. While the results are lower than those secured by the Van Slyke method, they are still sufficiently high to warrant the classing of these proteins with that group of proteins characterized by their high content of arginine, histidine, and lysine. Although more information is desirable concerning the basic amino acid content of the proteins synthesized from cystine, there is enough similarity between the data shown in Table IV to make it appear that the proteins are alike with respect to their content of the bases.

It has already been mentioned that the values for cystine shown in Table IV are open to question. There is apparently some difference of opinion regarding the presence of cystine in bacterial proteins. Tamura (11) was unable to detect any labile sulfur in either tubercle or diphtheria organisms grown on a medium containing peptone, while Johnson and Brown (12) recorded as much as 1 per cent of cystine nitrogen (as determined by the Van Slyke method) in tubercle bacilli cultured on a bouillon medium. Kishino (13) who also studied this question arrived at the conclusion that the proteins of *Bacillus coli* contained no cystine. Making use of the Okuda method, Kishino found appreciable amounts of cystine (0.9 per cent) in the proteins of two anaerobes, 0.2 per cent in those present in two aerobes, but none in the proteins of the colon bacillus. Our own results are not in concurrence with those mentioned above. The organisms grown on the cystine-free medium gave only the merest suggestion of a test for labile sulfur. The nitroprusside test as modified by Brand (14) and the Sullivan (15) test were both negative and no cystine was found by means of the Van Slyke method. On the other hand, a color like that given by cystine was obtained when the Folin and Marenzi (10) reagent was added to the hydrolyzed proteins. The blank, *i.e.* the color produced without the addition of sodium sulfite, was, however, quite high; in fact when this factor was taken into consideration, the amount of cystine in these proteins was almost negligible, being only 0.14 per cent. On the other hand, almost

all of the data secured in examining the products obtained from the cystine medium point to a synthesis of protein containing that amino acid.   The test for labile sulfur, as well as the nitroprusside test, was positive and the amount of cystine present, as determined by the Folin and Marenzi method, was 0.94 per cent.   The Sullivan reaction was likewise positive.

SUMMARY

1. It has been definitely established that *Bacillus coli* Jordan when grown in air can synthesize the amino acids arginine, histidine, and lysine from a medium containing only one source of nitrogen, namely *d*-alanine.

2. They can likewise synthesize tyrosine and tryptophane from media in which the sources of nitrogen are restricted to either *d*-alanine or *l*-cystine.

3. Presumptive evidence is available pointing to the synthesis of arginine, histidine, and lysine from a medium containing nitrogen only in the form of *l*-cystine.

4. No evidence of the synthesis of cystine from *d*-alanine was obtained.   Almost all of the data presented point to the synthesis of proteins containing cystine when that amino acid was added to the medium.

5. The lipids synthesized by the bacteria from "fat"-free media may be characterized by their low iodine numbers and by the absence of common sterols and fatty acids more highly unsaturated than oleic acid.

6. The colon bacilli developed on the alanine medium synthesized considerable amounts of the phospholipids, whereas those grown on a medium containing cystine had only a trace of such lipids.

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CONFIGURATIONAL RELATIONSHIP OF HYDROCARBONS

I. OPTICALLY ACTIVE METHANE DERIVATIVES CONTAINING PROPYL, ISOPROPYL, ISOBUTYL, ISOAMYL, AND ISOHEXYL GROUPS

BY P. A. LEVENE AND R. E. MARKER

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In a previous communication,[1] the conclusion was reached, on the basis of indirect evidence, that secondary carbinols of the isopropyl and of the isobutyl series are of opposite configurations when they rotate in the same direction. Furthermore, when both the isopropyl and the normal propyl series are arranged according to increasing molecular weights, then the members of the normal series above the symmetric member and the corresponding members of the isopropyl series are of opposite sign, whereas the members of the normal series below the symmetric member and the corresponding members of the isopropyl series are of the same sign. On the other hand, if the members of the isobutyl series and the corresponding normal series are arranged according to increasing molecular weights, then all the members above the symmetric member rotate in the same direction and all the members below in the opposite direction.

Work on the correlation of the carbinols of the isobutyl series by direct chemical means is in progress. Meanwhile, the task has been undertaken to compare the rotations of five hydrocarbons of identical configurations, methylethylpropylmethane, methylethylisopropylmethane, methylethylisobutylmethane, methylethylisoamylmethane, and methylethylisohexylmethane. Should the effect of the isopropyl and of the isobutyl groups be similar in the case of hydrocarbons to that observed in the case of secondary carbinols, then the configurationally related hydro-

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **90**, 669 (1931).

carbons containing the propyl and the isobutyl groups should rotate in the same direction whereas the hydrocarbon with the iso-propyl group should rotate in the opposite direction.

$$
\begin{array}{cccc}
\underset{|}{CH_3} & \underset{|}{CH_3}\ \overset{}{CH_3} & \underset{|}{CH_3}\ \ \ \overset{}{CH_3} & \underset{|}{CH_3}\ \ \ \ \ \overset{}{CH_3} \\
HC{-}C_3H_7 & HC{-}CH & HC{-}CH_2CH & HC{-}CH_2CH_2CH \\
\underset{|}{} & \underset{|}{}\ \ \ \diagdown & \underset{|}{}\ \ \ \ \ \diagdown & \underset{|}{}\ \ \ \ \ \ \ \ \ \diagdown \\
C_3H_6 & C_2H_6\ \ \ CH_3 & C_2H_6\ \ \ \ \ CH_3 & C_2H_6\ \ \ \ \ \ \ \ \ CH_3 \\
\text{Dextro} & \text{Levo} & \text{Dextro} & \text{Dextro}
\end{array}
$$
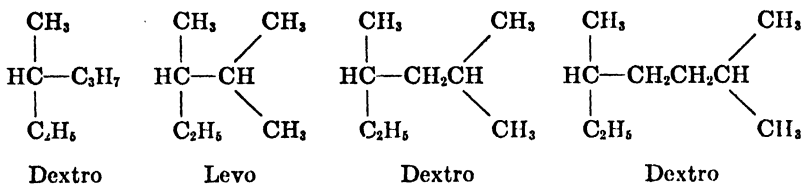
The identity of the configurations of the four hydrocarbons follows from the methods of their preparation. The two containing the propyl and isobutyl radicals may be traced back to the same dextro-2-ethylbutyric acid (4); the one containing the isoamyl group is derived from dextro-2-ethylvaleric acid (5), which is known to have the same configuration as dextro-2-ethylbutyric acid (4); finally, the configurations of the two hydrocarbons containing the propyl and the isopropyl groups are correlated by the fact that both have been prepared by condensation of the same methylethylbromomethane in one instance with malonic ester and in the other with methylmalonic ester. Hence, if a Walden inversion should have occurred in the last two reactions, it would have occurred in both in the same sense.

The progress of the synthesis of the hydrocarbons is given in Table I. From Table I it is seen that the directions of the rotations of the hydrocarbons containing the propyl, isopropyl, and isobutyl radicals are as was expected on the basis of the deductions made from observations on secondary carbinols containing the corresponding radicals.

The hydrocarbon with the isoamyl group was prepared in order to determine whether or not the differences in the effects of the isopropyl and isobutyl groups could be attributed to induced alternating polarity. In the case of the isopropyl derivative a methyl group is attached to the carbon atom adjacent to the asymmetric, in the isobutyl derivative on the second carbon atom from the asymmetric and in the isoamyl derivative on the third, hence again on the odd carbon atom. The direction of rotation of the isoamyl hydrocarbon was the same as that of the one containing the normal propyl radical. Whether the methyl group in the

TABLE I

$$[M]_D^{22°} \pm 2°$$

Isopropyl Series

$\begin{array}{c}CH_3\\HC{-}\\C_2H_5\end{array}$	$-OH$	$-Br$	$\begin{array}{c}CH_3\\ \diagdown\; H\\ -C-COOH\end{array}$	$\begin{array}{c}CH_3\\ \diagdown\; H\\ -C-COOC_2H_5\end{array}$	$\begin{array}{c}CH_3\\ \diagdown\; H\\ -C-CH_2OH\end{array}$	$\begin{array}{c}CH_3\\ \diagdown\; H\\ -C-CH_2Br\end{array}$	$\begin{array}{c}CH_3\\ \diagdown\; H\\ -C-CH\end{array}$
	$+6.19$	-18.89	$+1.56$	$+2.71$	-1.13	$+5.34$	-9.44

n-Propyl Series*

$\begin{array}{c}CH_3\\HC{-}\\C_2H_5\end{array}$	$-OH$	$-Br$	$-CH_2-COOH$	$-n-C_3H_7$
	$+4.91$	-14.81	$+2.85$	$+2.44$

Isobutyl Series†

$\begin{array}{c}CH_3\\HC{-}\\C_2H_5\end{array}$	$-OH$	$-Br$	$-CH_2-COOH$	$-CH_2-COOC_2H_5$	$\begin{array}{c}CH_3\\ -C-OH\\ -CH_2-\;\;CH_3\end{array}$	$\begin{array}{c}CH_3\\ -C-H\\ -CH_2-\;\;CH_3\end{array}$
			$+6.02$	$+6.73$	$+7.46$	$+12.37$
	$+4.91$	-14.81	$+2.95$			

Isoamyl Series

$\begin{array}{c}CH_3\\HC{-}CH_2{-}\\C_2H_5\end{array}$	$-Br$	$-CH_2-COOH$	$-CH_2-COOC_2H_5$	$\begin{array}{c}CH_3\\ -C-OH\\ -CH_2-\;\;CH_3\end{array}$	$\begin{array}{c}CH_3\\ -C-H\\ -CH_2-\;\;CH_3\end{array}$
	$+4\ 83$	$+8\ 11$	$+7\ 60$	$+5.31$	$+4\ 27$

* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).
† For convenience of discussion, the signs of rotation of all values in this series were changed from those found experimentally.

former hydrocarbon brings about a numerical change cannot be stated with certainty inasmuch as the maximum value of the rotation of the methylethyl-*n*-amylmethane is as yet not known. It seems, however, not improbable that numerically the value is smaller for the isoamyl derivative than for the normal amyl derivative.

The importance of the observations on these hydrocarbons, namely, on the propyl, the isopropyl, and the isobutyl derivatives, lies in the fact that they bring experimental support to the views previously expressed on the configurations of secondary carbinols of the isopropyl and of the isobutyl series.

The values of the rotations of the derivatives which led to the hydrocarbons can all be computed on a common basis. The basis for the computation is the values of the maximum rotations of the methylethyl carbinol and of the 2-ethylbutyric acid (4), which had been obtained by resolution. This permits the establishment of the ratio between the two values. Comparing the value of this ratio with that of the ratio between the rotation of the carbinol employed in our experiment and that of the 2-ethylbutyric acid derived from it, the degree of racemization of the 2-ethylbutyric acid obtained in our experiments can be calculated. The same degree of racemization may then be assumed to have taken place in the course of the reaction leading from the identical carbinol to 2-ethyl-3-methylbutyric acid (4). The reactions leading from the acids to the hydrocarbons proceed apparently without racemization inasmuch as in the case of the methylethyl-*n*-propylmethane the value obtained experimentally represents about 60 per cent of the value which is considered the maximum. With this assumption it is possible to calculate the maximum rotation of the methylethylisobutylmethane on the basis of the ratio between the maximum rotation of the 2-ethylbutyric acid (4) and the rotation of the acid employed by us. The value of the rotation of the methylethylisoamylmethane given in Table II may be regarded as the maximum value in view of the fact that the maximum rotation of the 2-ethylvaleric acid (5) is known. The methylethylisohexylmethane had been prepared by Kishner by reduction of citronellal and hence the value found by him may be regarded as the maximum value.

Table II contains the value of the maximum rotation of the

TABLE II

Calculated Maximum Molecular Rotations for Hydrocarbons

+10.25°*   +10.35°†   +9.5°‡   Maximum rotations from
                                               literature

$$
\begin{array}{ccc}
CH_3 & CH_3 & CH_2 \\
| & | & | \\
HC-OH \rightarrow & HC-CH_2-COOH \rightarrow & HC-n-C_3H_7 \\
| & | & | \\
C_2H_5 & C_2H_5 & C_2H_5
\end{array}
$$

+10.25°   +6.14°   +5.31°   Observed values

Calculated Maximum Rotations on Basis of Above Racemization

$$
\begin{array}{ccc}
CH_3 & CH_3 \quad CH_3 & CH_3 \quad CH_3 \\
| & | \quad | & | \quad | \\
HC-OH \rightarrow & HC-C-H & \rightarrow HC-C-H \\
| & | \quad \backslash & | \quad \backslash \\
C_2H_5 & C_2H_5 \quad COOH & C_2H_5 \quad CH_3
\end{array}
$$

+10 25°   +2.70°   −15.67°

$$
\begin{array}{cc}
CH_3 & CH_3 \qquad CH_3 \\
| & | \qquad | \\
HC-CH_2-COOH \rightarrow & HC-CH_2-C-H \\
| & | \qquad \backslash \\
C_2H_5 & C_2H_5 \qquad CH_3
\end{array}
$$

+10.35°   +21.28°

$$
\begin{array}{cc}
CH_3 & CH_3 \qquad CH_3 \\
| & | \qquad | \\
HC-CH_2-CH_2-COOH \rightarrow & HC-CH_2-CH_2-C-H \\
| & | \qquad \backslash \\
C_2H_5 & C_2H_5 \qquad CH_3
\end{array}
$$

+13.53°§   +7.09°‖

$$
\begin{array}{c}
CH_3 \qquad\qquad CH_3 \\
| \qquad\qquad | \\
HC-CH_2-CH_2-CH_2-C-H \\
| \qquad\qquad \backslash \\
C_2H_5 \qquad\qquad CH_3
\end{array}
$$

+8.90°¶
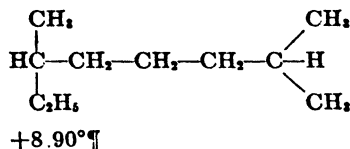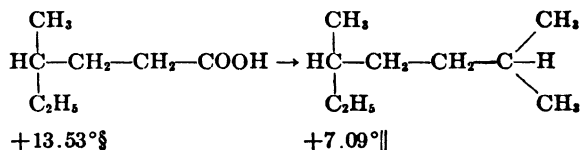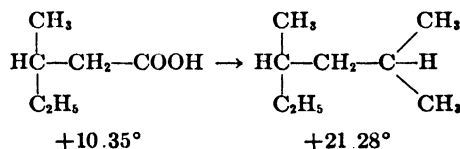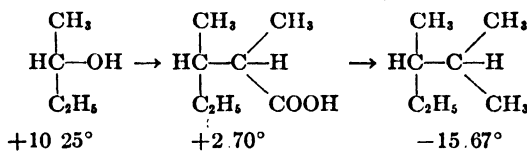
* Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **103**, 1940 (1913).
† Van Romburgh, P., *Rec. trav. chim. Pays-Bas*, **5**, 222 (1886).
‡ Welt, I., *Compt. rend. Acad.*, **119**, 745 (1894). Marckwald, W., *Ber. chem. Ges.*, **37**, 1046 (1904).
§ Walden, P., *Physik. Chem.*, **15**, 642 (1894).
‖ The value obtained by Welt is slightly higher than our experimental value due to impurities in her compound as is shown by her $D^{16.5}$ of 0 8813 which is far too high for a pure hydrocarbon and also by the boiling range of 6°.
¶ Kishner, *J. Russ. Physic.-Chem. Soc.*, **45**, 1786 (1913).

methylethylpropylmethane and of the corresponding isopropyl
and isobutyl derivatives calculated to the same basis.   As yet the
values cannot be regarded as final but imperfect as they are, they

TABLE III

Normal series	Iso series	Normal series	Iso series
C_3H_7 | HCOH | CH_3 Levo	C_3H_7 (i) | HCOH | CH_3 Dextro	C_4H_9 | HCOH | CH_3 Levo	C_4H_9 (i) | HCOH | CH_3 Levo
C_3H_7 | HCOH | C_2H_5 Levo	C_3H_7 (i) | HCOH | C_2H_5 Dextro	C_4H_9 | HCOH | C_2H_5 Levo	C_4H_9 (i) | HCOH | C_2H_5 Levo
C_3H_7 | HCOH | C_3H_7 0	C_3H_7 (i) | HCOH | C_3H_7 Dextro	C_4H_9 | HCOH | C_3H_7 Levo	C_4H_9 (i) | HCOH | C_3H_7 Levo
C_3H_7 | HCOH | C_4H_9 Dextro	C_3H_7 (i) | HCOH | C_4H_9 Dextro	C_4H_9 | HCOH | C_4H_9 0	C_4H_9 (i) | HCOH | C_4H_9 Levo
C_3H_7 | HCOH | C_5H_{11} Dextro	C_3H_7 (i) | HCOH | C_5H_{11} Dextro	C_4H_9 | HCOH | C_5H_{11} Dextro	C_4H_9 (i) | HCOH | C_5H_{11} Levo

may already warrant certain deductions.   Thus, comparing the
rotations of the propyl and the isopropyl hydrocarbons, a value of
25.2° is obtained.   It is noteworthy that the difference in the
values of the rotations of di-n-propyl carbinol (which is 0) and of

the propylisopropyl carbinol is 24.7°.   The coincidence is truly remarkable.   In order to make a similar estimate of the effect of the isobutyl group it would be necessary to compare the rotation of the methylethyl-*n*-butylmethane with that of the isobutyl hydrocarbon.   Unfortunately, the value of the maximum rotation of the methylethyl-*n*-butylmethane is as yet not available but with sufficient approximation for the present purpose, the rotation of the methylethylisobutylmethane can be compared with that of methylethyl-*n*-propylmethane.   This difference can be calculated from Table II to be 11.8°.   It is striking that this value is not far removed from the difference in the values of the rotation of di-*n*-butyl carbinol (which is 0) and of the *n*-butylisobutyl carbinol, which is 16.3°.   These numerical values, considered along with the values of the rotations of the carbinols given in Table III warrant definite conclusions regarding the factors which determine the value and the direction of the rotation of a given substance. Let us consider first the case of the two series of propyl and isopropyl carbinols.   Taking one pair, methylpropyl and methylisopropyl carbinols, both have one factor in common; namely, each is composed of radicals of the same molecular weight. Hence, contributions of the respective weights of the radicals is the same for both substances.   There is a second contribution to the molecular rotation of the isopropyl carbinol, namely that brought about by the presence of two methyl groups on the carbon atom adjacent to the asymmetric.   It is important to note that the reasoning which led to the correlation of the propyl and the isopropyl carbinols holds only if the isopropyl group is regarded as a propyl group in which the radical ethyl is replaced by two methyl groups and that the isobutyl, isoamyl, and isohexyl groups are derived in a similar manner and not by the introduction of an additional methyl group to the normal radicals containing one carbon less than is contained in the branched chain radical.   In other words, the changes do not produce a change in the molecular weights.   This circumstance more than anything else brings out the fact that the value and the direction of the rotation of a given molecule are brought about by the summation of several independent factors.   If the case of propyl and isopropyl secondary carbinols as well as the case of methylethyl-*n*-propylmethane and of methylethylisopropylmethane bring out the existence of two factors,

namely, of weight and of polarity (the isopropyl compared with propyl radical may be considered of higher polarity[2]), then the comparison of the series of methyl-*n*-butyl and methylisobutyl carbinols on one hand and of the configurationally related methylethylisopropylmethane, methylethylisobutyl-, methylethylisoamyl-, and methylethylhexylmethanes between themselves on the other hand bring out the effect of the distance of the polar group from the asymmetric carbon atom.   The effect of the distance already has been emphasized in connection with the effect of the double bond.   Whether or not an additional factor is introduced by the alternating induced polarity as yet cannot be stated definitely although the rotations of the three heavier hydrocarbons given in Table II seemed to suggest an affirmative answer to the question.

Incidentally, it may be mentioned that from the correlation of the hydrocarbons presented in Table II the configuration of citronellal is correlated with that of 2-ethylbutyric acid (4).

Future publications will deal with the rotatory contribution of other substituting groups.

<div align="center">EXPERIMENTAL</div>

Dextro-2-Bromobutane—50 gm. of 2-butanol, $[\alpha]_D^{25} = -6.63°$, were cooled in ice and saturated with hydrogen bromide.   The substance was then warmed on a steam bath for ½ hour, water was added, and the bromide separated.   It was shaken with cold concentrated sulfuric acid, washed with sodium carbonate solution, extracted with ether, and the ether solution dried with sodium sulfate.   Yield 64 gm.; b.p. 91° at 760 mm.; $D\dfrac{25}{4} = 1.251$.

$$[\alpha]_D^{25} = \frac{+13.52°}{1 \times 1.251} = +10.81°. \quad [M]_D^{25} = +14.81° \text{ (homogeneous)}$$

Levo-2-Ethylbutyric Acid (4) (β-Methylvaleric Acid)—12 gm. of sodium were dissolved in 120 cc. of absolute alcohol and 80 gm. of ethyl malonate were added followed by 63 gm. of *d*-2-bromobutane, $[\alpha]_D^{25} = +10.81°$.   The mixture was refluxed with stirring for 3 hours, then poured into water.   The ester was extracted with ether

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[2] Marshall, F. C. B., *J. Chem. Soc.*, 2754 (1930).

and distilled. The ester was hydrolyzed by refluxing 1 hour with 180 gm. of potassium hydroxide in 80 per cent alcohol. The alcohol was evaporated and the potassium salt acidified with dilute sulfuric acid. The malonic acid was extracted with ether, then decomposed by heating in a metal bath at 180° until carbon dioxide ceased coming off. It was then distilled under reduced pressure. The distillate was dissolved in sodium carbonate solution, extracted with ether, then acidified, and the organic acid extracted with ether. It was then distilled. Yield 22 gm.; b.p. 110° at 50 mm.; $D \dfrac{26}{4} = 0.923$.

$$[\alpha]_D^{26} = \frac{-2.35°}{1 \times 0.923} = -2.54°. \quad [M]_D^{26} = -2.95° \text{ (homogeneous)}$$

3.338 mg. substance: 7.605 mg. CO_2 and 3.130 mg. H_2O.
$C_6H_{12}O_2$. Calculated. C 62.02, H 10.42
Found. " 62.12, " 10.49

Levo-2-Bromobutane—200 gm. of 2-butanol, $[\alpha]_D^{25} = +8.37°$, were converted into the bromide as described for dextro-2-bromobutane. B.p. 91° at 760 mm.; yield 250 gm.; $D \dfrac{25}{4} = 1.251$.

$$[\alpha]_D^{25} = \frac{-17.25°}{1 \times 1.251} = -13.79°. \quad [M]_D^{25} = -18.89° \text{ (homogeneous)}$$

4.485 mg. substance: 5.805 mg. CO_2 and 2.625 mg. H_2O.
C_4H_9Br. Calculated. C 35.01, H 6.63
Found. " 35.29, " 6.55

Dextro-3,4-Dimethylvaleric Acid (5) (*Methyl-Sec-Butylacetic Acid*)—35 gm. of sodium were dissolved in 400 cc. of dry alcohol and 261 gm. of the ethyl ester of methylmalonic acid added. To the solution 195 gm. of 2-bromobutane of $[\alpha]_D^{25} = -13.79°$ were added. The mixture was refluxed with stirring for 3 hours, then cooled, and poured into water. The ester was extracted with ether, then distilled under reduced pressure. The ester was hydrolyzed by refluxing 1 hour with 275 gm. of potassium hydroxide in 80 per cent alcohol. The alcohol was evaporated and the residue acidified with dilute sulfuric acid. The malonic acid was

extracted with ether.   The ethereal solution was dried and the ether removed by distillation.   The acid was then heated in a metal bath at 190° until the evolution of carbon dioxide ceased. The product was then distilled under reduced pressure.   The distillate was dissolved in sodium carbonate solution and the solution was extracted with ether to remove impurities.   The carbonate solution was acidified and the organic acid extracted with ether, then distilled.   B.p. 92° at 15 mm.; yield 85 gm.; $D \frac{25}{4} = 0.921.$

$$[\alpha]_D^{25} = \frac{+ 1.24°}{1 \times 0.921} = + 1.35°.   [M]_D^{25} = + 1.56° \text{ (homogeneous)}$$

4.785 mg. substance: 11.390 mg. CO_2 and 4.670 mg. H_2O.
$C_7H_{14}O_2$.   Calculated.   C 64.62, H 10.84
Found.   " 64.91, " 10.92

Dextro-Ethyl Ester of 3,4-Dimethylvaleric Acid (5)—75 gm. of 3,4-dimethylvaleric acid (5), $[\alpha]_D^{25} = +1.35°$, were dissolved in 200 cc. of absolute alcohol containing 6 cc. of concentrated sulfuric acid.   The mixture was heated on a steam bath for 1 hour, cooled, and the excess alcohol evaporated.   The ester was shaken with dilute sodium carbonate solution, then extracted with ether, and distilled.   B.p. 78° at 22 mm.; yield 81 gm.; $D \frac{25}{4} = 0.875.$

$$[\alpha]_D^{25} = \frac{+ 3.28°}{2 \times 0.875} = + 1.88°.   [M]_D^{25} = + 2.71° \text{ (homogeneous)}$$

3.160 mg. substance: 7.905 mg. CO_2 and 3.270 mg. H_2O.
$C_9H_{18}O_2$.   Calculated.   C 68.29, H 11.47
Found.   " 68.21, " 11.58

Levo-2,3-Dimethyl-1-Pentanol—80 gm. of ethyl ester of 3,4-dimethylvaleric acid (5) were dissolved in 400 cc. of absolute alcohol and this was slowly dropped into a suspension of 161 gm. of sodium in 2 pounds of boiling toluene with rapid stirring.   The excess sodium was dissolved by adding additional alcohol.   The product was poured into water and extracted with ether, then fractionated.   The carbinol was purified through its half phthalic ester as previously described.[3]   B.p. 75° at 17 mm.; yield 45 gm.; $D \frac{23}{4} = 0.836.$

[3] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

$$[\alpha]_D^{33} = \frac{-0.81°}{1 \times 0.836} = -0.97°. \quad [M]_D^{33} = -1.13° \text{ (homogeneous)}$$

3.660 mg. substance: 9.715 mg. CO_2 and 4.430 mg. H_2O.
C$_7$H$_{16}$O. Calculated. C 72.5, H 13.8
Found. " 72.39, " 13.54

Dextro-1-Bromo-2,3-Dimethyl Pentane—40 gm. of 2,3-dimethyl-1-pentanol, $[\alpha]_D^{23} = -0.97°$, were cooled in ice and 60 gm. of phosphorus tribromide were added. This was then heated 1 hour on a steam bath, cooled, and poured on ice. The halide was separated and shaken first with cold concentrated sulfuric acid, then dilute sodium carbonate solution. It was dried with sodium sulfate and distilled. B.p. 67° at 25 mm.; yield 40 gm.; D $\frac{23}{4}$ = 1.166.

$$[\alpha]_D^{23} = \frac{+3.48°}{1 \times 1.166} = +2.99°. \quad [M]_D^{23} = +5.34° \text{ (homogeneous)}$$

5.910 mg. substance: 10.275 mg. CO_2 and 4.475 mg. H_2O.
C$_7$H$_{15}$Br. Calculated. C 46.90, H 7.99
Found. " 47.41, " 8.47

Levo-2,3-Dimethyl Pentane—A Grignard reagent was formed from 40 gm. of 1-bromo-2,3-dimethyl pentane, $[\alpha]_D^{23} = +2.99°$, and 5 gm. of magnesium in 100 cc. of dry ether. The Grignard reagent was poured into a mixture of ice and dilute hydrochloric acid. The hydrocarbon was extracted with ether and distilled. It was shaken with cold concentrated sulfuric acid, washed with sodium carbonate solution, and dried with sodium sulfate. It was refluxed with sodium for 2 hours, then distilled. B.p. 89–90° at 760 mm.; yield 12 gm.; D $\frac{21}{4}$ = 0.695.

$$[\alpha]_D^{n} = \frac{-6.56°}{1 \times 0.695} = -9.44°. \quad [M]_D^{n} = -9.44° \text{ (homogeneous)}$$

2.650 mg. substance: 8.170 mg. CO_2 and 3.820 mg. H_2O.
C$_7$H$_{16}$. Calculated. C 83.9, H 16.1
Found. " 84.07, " 16.12

Levo-Ethyl Ester of 2-Ethylbutyric Acid (4)—105 gm. of 2-ethylbutyric acid (4), $[\alpha]_D^{21} = -5.19°$, were mixed with 250 cc. of abso-

lute alcohol and 7 cc. of concentrated sulfuric acid, esterification being carried out as described for ethyl ester of 3,4-dimethylvaleric acid (5).   B.p. 68° at 25 mm.; yield 110 gm.; $D \dfrac{20}{4} = 0.878$.

$$[\alpha]_D^{20} = \dfrac{-4.11°}{1 \times 0.878} = -4.67°. \quad [M]_D^{20} = -6.73° \text{ (homogeneous)}$$

5.358 mg. substance: 13.110 mg. CO_2 and 5.235 mg. H_2O.
$C_8H_{16}O_2$.   Calculated.   C 66.62, H 11.17
Found.   " 66.72, " 10.93

Levo-2,4-Dimethyl Hexanol-2—50 gm. of ethyl ester of 2-ethyl-butyric acid (4), $[\alpha]_D^{20} = -4.67°$, were added to 2 mols of methyl magnesium iodide in dry ether.   The Grignard solution was decomposed and the carbinol obtained in the usual way.   B.p. 64° at 20 mm.; yield 41 gm.; $D \dfrac{21}{4} = 0.827$.

$$[\alpha]_D^{21} = \dfrac{-4.75°}{1 \times 0.827} = -5.74°. \quad [M]_D^{21} = -7.46° \text{ (homogeneous)}$$

2.972 mg. substance: 8.063 mg. CO_2 and 3.595 mg. H_2O.
$C_8H_{18}O$.   Calculated.   C 73.77, H 13.91
Found.   " 73.98, " 13.53

Levo-2,4-Dimethyl Hexane—25 gm. of 2,4-dimethyl hexanol were dehydrated by warming with a few crystals of iodine.[4]   The unsaturated hydrocarbon was refluxed and distilled from sodium. Yield 15 gm.   This unsaturated hydrocarbon was reduced by means of Adams' platinic oxide catalyst and hydrogen under 30 pounds pressure without solvent.   After adsorption ceased, the hydrocarbon was filtered from the catalyst, shaken with cold concentrated sulfuric acid, washed with sodium carbonate solution, and dried with anhydrous sodium sulfate.   It was then refluxed and distilled from sodium.   B.p. 110–111° at 760 mm.; yield 12 gm.; $D \dfrac{21}{4} = 0.703°$.

$$[\alpha]_D^{21} = \dfrac{-7.63°}{1 \times 0.703} = -10.85°. \quad [M]_D^{21} = -12.37° \text{ (homogeneous)}$$

[4] Edgar, G., and Marker, R. E., *J. Am. Chem. Soc.*, **51**, 1485 (1929).

3.763 mg. substance: 11.651 mg. CO_2 and 5.260 mg. H_2O.

C_8H_{18}. Calculated. C 84.13, H 15.87
　　　　Found.　　" 84.43, " 15.64

Dextro-3-Methylcaproic Acid (6)—25 gm. of sodium were dissolved in 250 cc. of absolute alcohol and 175 gm. of ethyl malonate were added. To the solution 140 gm. of 1-bromo-2-methylbutane, $[\alpha]_D^{22} = +3.90°$, were added. The acid was prepared as described for 2-ethylbutyric acid (4). B.p. 115° at 16 mm.; yield 70 gm.; $D \frac{22}{4} = 0.923$.

$$[\alpha]_D^{22} = \frac{+5.76°}{1 \times 0.923} = +6.24°. \quad [M]_D^{22} = +8.11° \text{ (homogeneous)}$$

4.476 mg. substance: 10.599 mg. CO_2 and 4.325 mg. H_2O.

$C_7H_{14}O_2$. Calculated. C 64.56, H 10.83
　　　　Found.　　" 64.57, " 10.81

Dextro-Ethyl Ester of 3-Methylcaproic Acid (6)—70 gm. of 3-methylcaproic acid (6) were dissolved in 200 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. Esterification was carried out as described for the ethyl ester of methyl-sec-butyl-acetic acid. B.p. 80° at 20 mm.; yield 65 gm.; $D \frac{22}{4} = 0.888$.

$$[\alpha]_D^{22} = \frac{+4.27°}{1 \times 0.888} = +4.81°. \quad [M]_D^{22} = +7.60° \text{ (homogeneous)}$$

$C_9H_{18}O_2$. Calculated. C 68.29, H 11.47
　　　　Found.　　" 68.23, " 11.52

Dextro-2,5-Dimethyl Heptanol-2—To 2 mols of methyl magnesium iodide in ether were added 65 gm. of the ethyl ester of 3-methylcaproic acid (6), $[\alpha]_D^{22} = +4.81°$. The Grignard solution was poured on ice and ammonium chloride. The reaction product was extracted with ether. The ethereal solution was dried with anhydrous sodium sulfate and distilled. B.p. 75° at 15 mm.; yield 48 gm.; $D \frac{22}{4} = 0.830$.

$$[\alpha]_D^{22} = \frac{+3.07°}{1 \times 0.830} = +3.69°. \quad [M]_D^{22} = +5.31° \text{ (homogeneous)}$$

4.957 mg. substance: 13.655 mg. CO_2 and 6.045 mg. H_2O.
$C_9H_{20}O$.   Calculated.   C 74.89, H 14.01
Found.*   " 75.12, " 13.64

Dextro-2,5-Dimethyl Heptane—40 gm. of 2,5-dimethyl heptanol-2 were dehydrated with iodine as described under 2,4-dimethyl hexane. The unsaturated product was distilled from sodium. 15 gm. of this material were reduced with hydrogen in the presence of 1 gm. of Adams' platinic oxide catalyst at 30 pounds pressure. The hydrocarbon was purified as described for 2,4-dimethyl hex-

ane.   B.p. 135° at 760 mm.; yield 8 gm.; D $\dfrac{22}{4}$ = 0.714.

$$[\alpha]_D^{22} \quad \dfrac{+\ 2.38°}{1 \times 0.714} = +\ 3.33°. \quad [M]_D^{22} = +\ 4.27° \text{ (homogeneous)}$$

2.625 mg. substance: 8.170 mg. CO_2 and 3.640 mg. H_2O.
C_9H_{20}.   Calculated.   C 84.24, H 15.76
Found.   " 84.55, " 15.46

Dextro-n-Propyl-n-Butyl Carbinol—The inactive carbinol was prepared from n-butyl magnesium bromide and n-butyl aldehyde. This was converted into its half phthalic ester and resolved by crystallizing its strychnine salt from acetone until the rotation of the phthalate reached a constant value.[1]   This rotation was obtained after six crystallizations.

$$[\alpha]_D^{22} = \dfrac{+\ 0.55° \times 100}{1 \times 24.2} = +\ 2.28° \text{ (in absolute alcohol)}$$

The phthalic ester was hydrolyzed by sodium hydroxide and the

carbinol distilled.   B.p. 79° at 16 mm.; D $\dfrac{22}{4}$ = 0.818.

$$[\alpha]_D^{22} = \dfrac{+\ 0.61°}{1 \times 0.818} = +\ 0.74°. \quad [M]_D^{22} = +\ 0.95° \text{ (homogeneous)}$$

This resolution was repeated with brucine instead of strychnine and the same value was obtained.

3.330 mg. substance: 9.015 mg. CO_2 and 4.125 mg. H_2O.
$C_8H_{18}O$.   Calculated.   C 73.77, H 13.91
Found.   " 73.82, " 13.86

SOME FACTORS INFLUENCING THE ACTIVITY OF PEROXIDASE*

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Of the reactions in which catalysis is due to enzymes, those involving oxidation and reduction have received far less attention than have the hydrolytic type. The following investigation was undertaken in order to determine some of the factors which influence the reactions of the peroxidases.

Preparation of the Enzyme

An important source and preparation method are reported from the Munich laboratories of Willstätter (1–3) and his associates who have prepared a highly purified peroxidase from horseradish. The methods of Willstätter, with certain modifications, were employed in the present investigation.

About 14 pounds of horseradish roots were cleaned, sliced thin, and self-dialyzed in running water for 8 days. The slices darkened and, in confirmation of Willstätter's statement, those which developed a deep brown color throughout the slice gave the best yields. After dialysis the slices were freed from water; the enzyme was "set" with oxalic acid; the solid was finely ground, and the enzyme was extracted with barium hydroxide. The enzyme solution was partially purified by treatment with carbon dioxide and with nine-tenths its volume of absolute alcohol and the solution rapidly concentrated below 35° *in vacuo* from 1400 cc. to

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about 75 cc. The residual yellow liquid, after clarification in a centrifuge, was treated at 10° with 5 volumes of absolute alcohol, resulting in precipitation of the easily filtrable enzyme. It was further purified with dilute sulfuric acid, twice reprecipitated with absolute alcohol, and an aqueous stock solution prepared. This final solution was kept at a low temperature and largely diluted for use.

Composition of the Enzyme Material

The solid enzyme material was of a light cream color and contained 5.8 per cent ash. Its response was negative to the usual protein tests, except for a weak xanthoproteic reaction. The ash contained iron but gave a negative test for manganese by the Marshall persulfate method. A purpurogallin number of 205 was obtained when the enzyme was evaluated according to Willstätter's method (1).

Effect of Hydrogen Ion Concentration

For establishing the various hydrogen ion conditions, investigators have used organic acids and their salts, mineral acids, ammonia, and the alkali hydroxides, carbonates, and phosphates. In a very extended study of malt amylase, Sherman (4) employed combinations of phosphoric acid, its sodium salts, sodium carbonate and sodium hydroxide. These substances were selected for the present study. Their solutions were prepared from the purest samples obtainable. For enzyme solutions, the stock sample was largely diluted to produce a concentration which would form a convenient amount of product. Resublimed pyrogallol was dissolved in redistilled water to a concentration of 20 gm. per 100 cc. A fresh solution was prepared daily. Merck's superoxol (30 per cent hydrogen peroxide) was distilled *in vacuo*, analyzed, and diluted to a 2 per cent solution. For the color comparison solution, pure purpurogallin was prepared by the method of Perkin and Steven (5). A standard solution of suitable color intensity was secured by employing a concentration in ether of 50 mg. per liter. Since it was not feasible to use an ether solution in actual measurements, the color was duplicated by a 0.034 per cent aqueous solution of potassium dichromate. The latter solution constituted the color standard. Pure, iron-free, 10 per cent sulfuric acid was
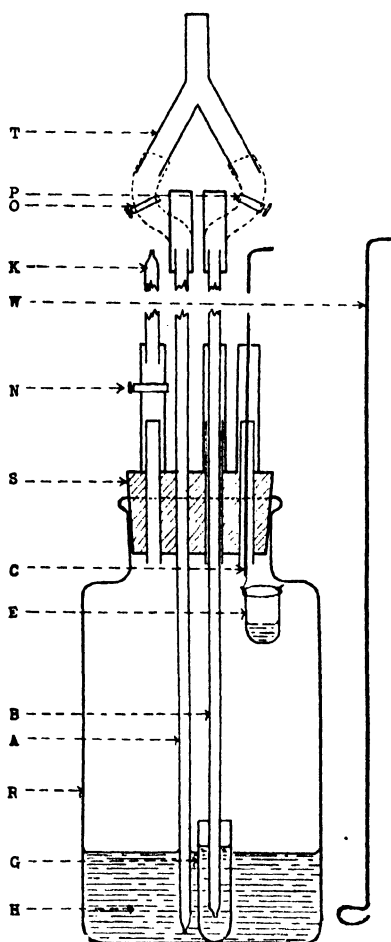
Fig. 1. Chamber for peroxidase reaction in alkaline solutions. R represents the reaction bottle; A and B, tubes for introducing nitrogen; T, Y-tube for connecting to nitrogen supply; P, O, and N, screw clamps for closing system after disconnecting from nitrogen supply; E, capsule containing enzyme; C, sliding wire support for capsule E; G, capsule containing pyrogallol; W, wire for lowering capsule G; H, hydrogen ion reagent in measured volume of water; K, capillary outlet to relieve pressure during nitrogen charging; and S, stopper supporting tube system.

employed. The ether and diluting water were redistilled for use. For acid solutions, measurements were made in an atmosphere of air; for basic solutions, a nitrogen atmosphere was very successfully employed.

TABLE I

Effect of Hydrogen Ion Concentration on Activity of Peroxidase

Hydrogen ion reagent	Concentration in reaction mixture	Purpurogallin	pH
	μ	mg.	
H_3PO_4	0.00045	1.1	3.3
"	0.00030	1.1	3.4
"	0.00015	1.5	3.7
NaH_2PO_4	0.040	2.3	4.4
NaH_2PO_4	0.012	2.5	4.4
H_3PO_4	0.00008		
NaH_2PO_4	0.006	3.4	4.4
H_3PO_4	0.000065		
NaH_2PO_4	0.0025	3.6	4.8
"	0.0005	3.7	5.1
None		5.5	6.0
Na_2HPO_4	0.001	8.2	7.3
Na_2HPO_4	0.005	8.1	7.5
Na_2CO_3	0.00024		
Na_2HPO_4	0.005	9.0	7.8
Na_2CO_3	0.00084		
NaOH	0.001	8.7	8.0
Na_2CO_3	0.0016	8.4	8.3
NaOH	0.0015	5.2	8.4
"	0.002	4.5	8.5
"	0.006	4.4	9.0
"	0.010	0.0	10.6

All reactants were measured directly from the ice bath. Into the measuring flask were introduced 5 cc. of pyrogallol solution, 1 cc. of hydrogen peroxide, and the desired volume of hydrogen ion reagent; the whole was diluted with water to 99 cc. After

thorough mixing the solution was cooled to 0° in the reaction flask and 1 cc. of the enzyme was added in a small capsule. The reaction was allowed to proceed for exactly 12 minutes, then arrested by the addition of 5 cc. of sulfuric acid. The purpurogallin was next extracted in a separatory funnel with four portions of ether and the combined extracts made up to 50 cc. A sample of this extract was colorimetrically compared with the standard in a series of ten readings per determination.

For reactions in a basic solution, the above process was modified slightly (Fig. 1). The hydrogen ion reagent, with the measured volume of water (H), was introduced into the 500 cc. wide mouth reaction bottle (R), which was fitted with a 4-hole rubber stopper (S). The enzyme solution was placed in the capsule (E), supported in a loop of light copper wire attached to a heavy wire (C) which could be raised and lowered through an air-tight rubber collar in the stopper. The pyrogallol was contained in a larger capsule (G). The system was charged with purified nitrogen through the Y-tube T and tubes A and B, the pressure being relieved through capillary K. After the oxygen was entirely swept out, the apparatus was disconnected, the contents of the capsule (G) were emptied by raising tube B, the system was cooled to 0°, and the enzyme was finally introduced by lowering capsule E. After the reaction had proceeded for exactly 12 minutes, sulfuric acid was introduced through tube B and the product was extracted with ether and measured as described before.

As a method of determining hydrogen ion concentrations, solutions were prepared duplicating those mentioned above but without the 1 cc. of enzyme solution. The presence of the enzyme, with the resulting oxidation of pyrogallol, produced constantly changing values. It was determined, however, in the more strongly acid solutions where enzyme activity is low, that the presence of the purified enzyme solution did not appreciably modify the hydrogen ion concentration. The quinhydrone electrode was used for the acid region and the hydrogen electrode for the basic solutions.

In Table I the activity of the peroxidase is expressed in terms of mg. of purpurogallin formed for each concentration of hydrogen ions. On account of the catalysis of hydrogen peroxide by bases, it was necessary to run blank determinations on the basic

solutions in order to measure the amount of purpurogallin so produced. Such corrections are included in the data of Table I. Blank determinations on the acid mixtures gave negative results. The phosphate ions are not without influence on the reaction rate, but their effect is minor. This is borne out by the work of Smirnow (6) who observed that phosphates exerted but little influence on crude peroxidase of wheat. No hydrogen ion reagent could be selected for these measurements which would not produce some effect of its own. It would not be permissible, however, to assume that the effects of the enzyme and reagent are additive and correct the data accordingly. The 12 minute reaction time was selected as best under the conditions of experimentation. A shorter period introduced too high a percentage of error and a much

TABLE II

Effect of Enzyme Concentration on Yield of Product

pH 4.8.

Enzyme concentration	Purpurogallin formed
mg.	*mg.*
0.14	0.2
0.28	1.4
0.42	2.8
0.57	3.1
0.85	6.7
1.70	13.2

longer time unnecessarily prolonged the measurements. In most of the reaction mixtures a typical yellow color was developed during the 12 minute period, but varying in shade with the reaction of the solution. The ether extract was yellow and the aqueous extraction residue was colorless or pale pink. 65 cc. of ether, added in four portions as described, proved to be the most efficient practice for the complete extraction of the purpurogallin. Nearly 20 cc. were miscible with the water, leaving the total volume of the extracts a little less than 50 cc. Duplicate determinations usually gave excellent checks, the colorimeter average readings varying by not more than ±0.2 mm. With a view to extending the length of the reading day, various illuminating devices were tested with the colorimeter. In no case would artificial lighting afford

the reading accuracy attainable with north sky light. The point of inactivity in the basic region is, by extrapolation on a curve, too far into the basic field. Inasmuch as this relatively high concentration of base resulted in the formation of other colored products, an accurate determination of the exact point of inactivation was not possible.

Effect of Concentration of the Enzyme

Although enzymatic concentration has no effect on the final reaction equilibrium, it does influence reaction velocities. In order to study this factor in relation to peroxidase, various concentrations of the enzyme were evaluated by the method already described, all other factors being maintained constant. The results are given in Table II. Since we possess no generally accepted explanation of the mechanics of enzymatic oxidations, a discussion of the effect of the concentration of a given enzyme can do no more than establish the velocity values for the system under consideration. An explanation advanced by Willstätter and Weber (7) assumes, for peroxidase, the formation of two addition compounds, *viz.* $RHO \cdot OH$ and $RH_2 \ O:O$, the one active and the other inactive. At any rate, preponderance of opinion seems to favor the view that the substrate is oxidized by activated oxygen formed through union of the enzyme with the peroxide.

Effect of Concentration of Substrate

This relationship has been most widely studied in connection with the hydrolysis of sucrose by the invertase of yeast. For that enzyme Nelson and Schubert (8) report a maximum yield at a 5 per cent substrate concentration. For the data of peroxidase recorded in this paper, concentrations of pyrogallol were employed ranging from 0.2 to 5 gm. per 100 cc. The enzyme was added to the solutions containing this substrate and hydrogen peroxide, and evaluated as usual. In Fig. 2 are shown graphs of the results.

The decreased activity at low concentrations is probably a mass action effect. It is not due to lack of sufficient pyrogallol to produce maximum yield. The falling off of the curve from the point of maximum activity can best be explained as a reduction in the active surface of the enzyme, brought about by the formation of an

addition compound with the large excess of the pyrogallol. The hydrogen ion concentration of all of the solutions was so nearly constant (pH 6.1 ± 0.1) that this factor played very little part in the variations of enzymatic activity. The concentration for maximum activity is that which was employed throughout this research.
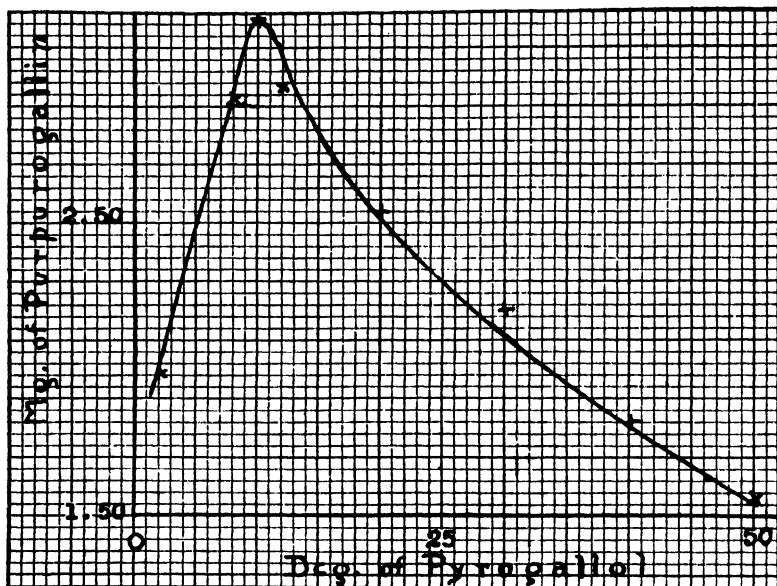
FIG. 2. Effect of concentration of substrate on activity of peroxidase

Effect of Salts

The presence of salts in all enzymatic processes of the animal organism renders the study of their influence of especial interest. Solutions of pure salts, whose concentrations in each reaction mixture are indicated in the data, were added to the pyrogallol-hydrogen peroxide mixture; the enzyme was introduced and the reaction followed as described before. For the first series, several concentrations of each salt were used. Blanks were run on each sample and if the salt alone was thus shown to catalyze the reac-

tion, its effect is indicated in Table III under "Activity of salt alone." When the enzyme was used with a catalytically active salt, it could not be assumed that their combined effect was addi-

TABLE III

Effect of Different Concentrations of Various Salts on Activity of Peroxidase

Salt	Concentration in reaction mixture	Activity of salt alone	Activity of enzyme and salt	pH
	M	*per cent*	*per cent*	
Ferric chloride	0.0000001	0	100	4.8
	0.0000005	2.6	95	
	0.000001	3.4	90	
Manganese sulfate	0.0001	0	86	5.9
	0.001	0	74	
Copper sulfate	0.00001	0	100	
	0.0001	2.5	75	4.8
	0.0005	5.0	63	
Mercuric chloride	0.0000001	0	100	
	0.000001	0	99	
	0.00001	3.3	68	
	0.0001		10	4.9
Sodium chloride	0.0001	0	92	6.0
	0.001	0	84	
	0.01	0	57	
Sodium tungstate	0.000001	0	89	5.9
	0.0001	Very high		
Uranium chloride	0.000001	0	83	5.7
	0.0001	0	11	
	0.0003	0	0	
Thorium nitrate	0.0000001	0	95	5.2
	0.000001	0	54	
	0.0001	0	0	
Potassium cyanide	0.0000001	0	99	
	0.000001	0	66	6.1
	0.00001	0	2	6.8
	0.0001	0	2	8.5

tive. Hence the data for their effect, recorded under "Activity of enzyme and salt," are not corrected for the influence of the salt alone. In Table III and subsequent tables the term "percentage activity" is used to indicate the catalytic power of the enzyme under the designated experimental conditions. The actual weight of the purpurogallin produced could not serve as a basis of comparison because the activity of the enzyme did not remain constant throughout the period of the investigation. The percentage activity was computed by dividing the weight of purpurogallin formed under the modified, designated conditions of each experiment by the weight formed by the enzyme alone in the absence of such disturbing factors (Table III).

A few comments on Table III are pertinent. In a solution of pH 6, with a 0.000001 M concentration of ferric chloride, the salt alone showed no catalyzing effect, but the percentage activity of the enzyme in its presence dropped to 79.5 per cent. When 68 mg. of the enzyme were mixed with 0.00001 mol of ferric chloride in a volume of 8 cc. and allowed to stand for 30 minutes, the salt was shown to be completely adsorbed from the solution and the activity of the enzyme was reduced 6 per cent. When measurements were made with a copper sulfate concentration of 0.0001 M but at a pH 5.9, the percentage activity of the enzyme dropped from 75.0 to 22.9. In another study on the influence of copper, samples of the enzyme were mixed with the copper sulfate solution of 0.0001 M concentration 5 hours and 12 hours before being subjected to measurement. The results were as follows:

Treatment	Percentage activity
Mixed and measured at once	75.0
After 5 hrs.	71.7
" 12 "	37.8

When the 0.0005 M concentration stood in contact with the enzyme for 12 hours, the percentage activity dropped from 63 to 27.0. Hence the toxicity of copper sulfate increases with the concentration of the salt, with decreasing acidity of the solution, and with increasing time of preliminary contact of enzyme and salt. Contact of the uranium salt with hydrogen peroxide produced a gradual separation of a peruranate (9), hence the effective mass of uranium ions was exceedingly small. This may account for the apparently greater inactivating effects of thorium.

Effect of Anions

Soluble fluorides are harmful to many enzymes. When applied to peroxidase, 0.0001 mol of sodium fluoride, at pH 6, reduced the percentage activity to 27. In the case of potassium cyanide the poisonous action was even more marked (Table III). Its pH value of 6.8 would, of itself, correspond to high activity, yet the potassium cyanide actually produced almost complete inactivation. The sulfide anion proved highly toxic. When 1 cc. of the enzyme solution was saturated with purified hydrogen sulfide and the latter subsequently removed by aeration, the percentage activity was

TABLE IV

Effect of Single Concentrations of Various Salts on Activity of Peroxidase

Salt	Concentration in reaction mixture	Net activity	pH
	M	*per cent*	
Lead nitrate	0.0001	60	5.6
Cobalt chloride	0.0001	67	6.0
Nickel "	0.0001	74	6.1
Chromous "	0.0001	84	4.6
Aluminum sulfate	0.0001	88	4.3
Barium chloride	0.0001	74	6.2
Calcium "	0.0001	80	6.2
Magnesium sulfate	0.0001	82	6.2
Sodium chlorate	0.0001	93	6.1
" nitrate	0.0001	97	6.2
" sulfate	0.0001	97	6.2
	0.001	88	6.2
Potassium chloride	0.0001	98	6.2

only 16. The enzyme was completely inactivated by treatment with 0.00001 mol of potassium sulfide at pH 7.5. Treatment of the enzyme with the platinum poisons, carbon monoxide and arsine, for 10 minutes reduced the percentage activity to 91.5 and 86.2, respectively.

For the second series of salt studies, salts were selected which proved to be without catalytic effect upon a hydrogen peroxide-pyrogallol mixture at the concentrations employed. Hence their influence upon the catalytic action of the peroxidase would be due to the specific action of the salt itself and to the hydrogen ion concentration which it established. These two influences may not be

purely additive but it appeared that a closer evaluation of the specific salt effect might be arrived at by considering them as additive. The pH of the solutions containing the neutral salts was 6.2 and in all other cases less than 6.2. Hence the purpurogallin yield at pH 6.2 was taken as the standard, and in the "Net activity" of the enzyme in the presence of the salt (Table IV), correction has been made for any reduction in the activity of the enzyme due to a pH of less than 6.2.

Effect of Various Organic Compounds

Absolute ethyl alcohol was mixed in varying proportions with the reaction mixture, producing inactivations proportional to its concentration (Table V). It is considered that inhibition by

TABLE V

Effect of Alcohol on Activity of Peroxidase

Alcohol ratio	Activity
	per cent
5:95	95
20:80	76
30:70	42
50:50	0.5

alcohol is due both to a reduction in the degree of dispersion of the enzyme and to its actual destruction.

The glucoside of horseradish is sinigrin, not obtainable on the market. Peroxidase was unaffected by five other glucosides: amygdalin (0.00001 mol), and digitalin, saponin, salicin, and strophanthin (each 0.00005 mol).

The susceptibility of the enzyme to alkaloids was examined. It was not affected by caffein (21 mg.), atropine (69 mg.), cinchonidine sulfate (48 mg.), quinidine sulfate (8 mg.), strychnine sulfate (17 mg.), and quinine bisulfate (55 mg.). Allowance being made for its hydrogen ion concentration, nicotine (16 mg. or 0.0001 mol) produced an acceleration of 44 per cent. Unless this alkaloid is specific, some undetermined factor must be operative.

A number of organic compounds were selected for study because of their wide distribution in cell contents and plant structure. Glucose (90 mg.), sucrose (34 mg.), and soluble starch (50 mg.)

produced no effect. Purified gelatin (32 mg.) and egg albumin (32 mg.) had no pronounced influence but their emulsifying action prevented quantitative evaluation. With allowance for hydrogen ion effects, dextrin (32 mg.) reduced the activity 33 per cent and tannic acid (32 mg.) 55 per cent. Reduction of the active surface of the enzyme by adsorption on the colloidal dextrins could account for their inhibiting influence. Since tannic acid is a strong reducing agent it, as well as the pyrogallol, may be an oxygen acceptor in the enzyme catalysis, thus reducing the amount of oxidation product of the latter. The convenient compound formation theory offers another explanation for the inhibiting effect of tannic acid.

Effect of Physical Factors

Irradiation—1 cc. of enzyme, both alone and when contained in the diluting water, was exposed in quartz for 10 minutes to ultra-violet light. The volume of water reduced the destructive action of the rays.

Treatment	Percentage activity
Irradiation in 94 cc. water	13.5
" " 1 " "	0.0

It is generally observed that ultra-violet light is more potent in enzyme destruction than x-rays, radium, or visible light.

Aeration—The method of aeration consisted of bubbling the gas from the tip of a very fine capillary tube through 1 cc. of the enzyme contained in the capsule. The latter was then introduced into the reaction mixture. In one series filtered air was used, in another, pure oxygen, in a third, carbon dioxide followed by pure oxygen to sweep out the former. In each case the percentage activity was reduced to about 85. An extension of pure oxygen aeration from the 10 minute period to 20 minutes did not modify the value. This effect may be due to the oxidation of some component of the enzyme system or to a reduction in the degree of dispersion of the colloidal enzyme similar to the flocculating effect of gases upon ferric oxide sols, as observed by Stark (10).

Dialysis—The purpose of this study was (a) to examine the effect of membranes upon the enzymatic activity and (b) to determine whether the poisoning effect of heavy metal salts, *e.g.* mercuric chloride, is exerted directly upon the enzyme. For the first study,

small dialysis sacs of collodion, of parchment, and of viscose were prepared, thoroughly washed, and charged with 1 cc. of the enzyme solution. After dialysis in running distilled water they were rinsed into the reaction capsule and evaluated. The data in Table VI indicate reduced activity with each membrane, increasing with time of contact. Supplementary determinations also established the following facts: (1) no enzyme passed through the membranes; (2) percentages of recovery of the enzyme in the transfer process alone were for collodion 100, viscose 100, parchment 96.5. Adsorption of the enzyme by the colloidal membranes, thus reducing its active surface, explains the inactivation effects.

In the second study, mixtures of 1 cc. of enzyme and 1 cc. of mercuric chloride (2.7 mg.) were dialyzed in collodion and exam-

TABLE VI

Effect of Dialysis Membranes on Activity of Peroxidase

Membrane	Dialysis	Activity
		per cent
Collodion	1 day	39
Parchment	30 min.	67
	4 hrs.	55
	2 days	7
Viscose	30 min.	87
	3 hrs.	75
	2 days	36

ined at the end of 1 and of 2 days. The percentage activity values were at the end of 1 day 20.0, at the end of 2 days 7.7. Other samples so dialyzed showed no mercury ions in the mixture at the end of the 1st day. Reference to Table VI will show that the mercuric chloride inactivation is higher than the membrane alone could accomplish. Hence the toxic action of mercuric chloride is, in part at least, exerted directly upon the enzyme.

Temperature Coefficient—Measurements were conducted as usual, with thermostat-controlled baths. Since it was not convenient to remove samples for test at frequent intervals, the quantities of purpurogallin formed in 12 minutes at the different temperatures were taken as a measure of the enzymatic activity or velocity. In the 12 minute period the weight of product formed

at 10° was 140 per cent of that formed at 0°, and at 20° the product was 120 per cent of that formed at 10°. Hence the temperature coefficient was 1.4 in the 0–10° range and in the 10–20° interval it was 1.2. A correction for heat decomposition effects upon hydrogen peroxide is incorporated in the 20° value. At higher temperatures, 50°, 40°, and even 30°, thermal decomposition of hydrogen peroxide and of the enzyme rendered quantitative data undependable.

SUMMARY

1. Modifications in the methods of preparing and evaluating peroxidase have been developed.

2. The effects on the activity of peroxidase of varying concentrations of hydrogen ion, substrate, and enzyme have been measured.

3. The activity of peroxidase has been evaluated after subjecting it to irradiation, to aeration, to different reaction temperatures, and to several dialysis membranes.

4. Data are reported for the catalytic activity of peroxidase in the presence of a wide variety of inorganic and organic compounds.

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THE OXIDATION OF COBALTOUS CYSTEINE

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Michaelis and Barron (13) have shown that cobaltous salts react with cysteine and that the product of this reaction can be oxidized to a brown cobaltic cysteine complex. Through the use of three oxidizing agents: air, ferricyanide, and phenolindophenol, they concluded that 1 atom of cobalt reacts with 3 molecules of cysteine and that 2 equivalents of an oxidizing agent are required to form each molecule of the complex. Michaelis and Yamaguchi (14) suggested that the reaction which leads to the formation of the brown complex may be written: "$CoX_2 + 3\ HSCH_2 \cdot NH_2CH \cdot COOH + \frac{1}{2} O_2 \rightarrow Co(SCH_2 \cdot NH_2CH \cdot COO)_3H_2 + 2XH + H_2O$ where X is any ordinary monovalent acid rest or OH."

The oxidation of cobaltous cysteine has been further studied with nine oxidizing agents. These results show that there is a high degree of specificity in the reaction with each oxidant. It therefore becomes necessary to determine whether some portion of the oxidants used by Michaelis and coworkers could have brought about reactions other than the oxidation of cobaltous cysteine to the brown complex. It would seem a remarkable coincidence if such reactions could occur and nevertheless have the amount of all three oxidants remain equal to two-thirds the concentration of the thiol group. The results of the experiments to be recorded, however, indicate that such is the fact. The apparent quantitative agreement between the three oxidants used by Michaelis and coworkers has been shown to be due to coincidence. There are three different side reactions which occur with the three different oxidizing agents.

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Oxidation of Cobaltous Cysteine with Indigo Disulfonate

Kendall and Nord (11) have shown that the thiol group of cysteine cannot reduce indigo disulfonate. Michaelis and Barron (13) found that this dye is reduced by cobaltous cysteine, but they did not determine the quantitative relationship between cobaltous cysteine and this oxidizing agent.
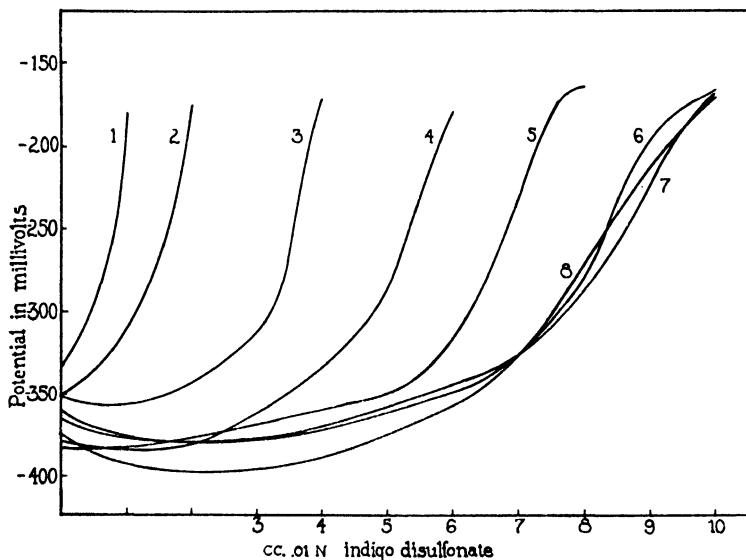
Fig. 1. Oxidation of a constant amount of cysteine in the presence of varying amounts of cobalt with indigo disulfonate. Each of the solutions represented by the curves contained 2×10^{-4} mols of cysteine and cobalt sulfate in the following amounts: 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.5 × 10^{-4} mols. Curve 6 represents the amounts of cysteine and cobalt which are in a ratio of 2:1. Although cobalt was present in excess of this amount in electrode Cells 7 and 8 (Curves 7 and 8), the amount of oxidant reduced was not increased.

We have found that regardless of the relation between the amounts of cysteine and cobalt 2 molecules of cysteine combine with 1 atom of cobalt and require 1 equivalent of an oxidant to form the brown cobaltic complex. If the mols of cobalt exceed a half the number of mols of cysteine, the number of equivalents of indigo disulfonate required for complete oxidation of the cobaltous

cysteine is equal to a half the number of mols of cysteine.   If the mols of cobalt are less than a half the number of mols of cysteine, the number of equivalents of indigo disulfonate required is equal to the number of mols of cobalt.

If the mols of cysteine exceed twice the number of mols of cobalt, indigo disulfonate may oxidize a small amount of the cysteine to cystine.   With all other proportions between cysteine and cobalt
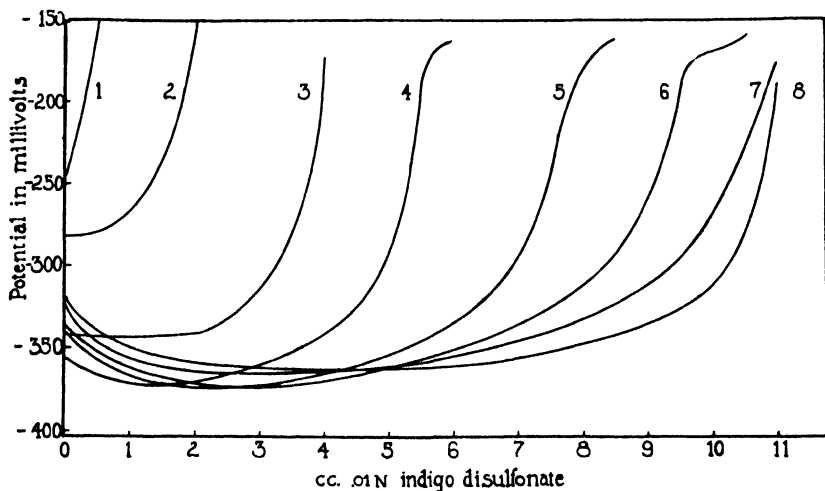
FIG. 2. Oxidation of varying amounts of cysteine in the presence of a constant amount of cobalt with indigo disulfonate.   Each of the solutions represented by the curves contained 1×10^{-4} mols of cobalt sulfate and cysteine in the following amounts: 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, and 3.0 $\times$ 10^{-4} mols.   Electrode Cell 6 (Curve 6) contained cysteine and cobalt in the ratio of 2:1.   Slightly increased amounts of indigo disulfonate were reduced in Cells 7 and 8 (Curves 7 and 8) by the oxidation of a small amount of the cysteine to cystine.

titration with indigo disulfonate is an accurate determination of the amount of cobaltous cysteine which is present in solution. See Figs. 1, 2, and 11.

The amount of indigo disulfonate required for the complete oxidation of cobaltous cysteine was determined potentiometrically. The end-point which was taken to indicate complete oxidation of the cobaltous cysteine was a decrease in the reduction potential to -0.168 volt.   If the ratio of cysteine to cobalt is 2:1, neither

cystine nor cobalt ion is present in solution after oxidation with indigo disulfonate.

When the cobaltic cysteine complex has once been formed it is stable in the presence of ferricyanide. If cobaltous cysteine is oxidized with indigo disulfonate to the brown complex the addition of ferricyanide will oxidize the indigo white disulfonate but as soon as a volume of ferricyanide which is just equivalent to the indigo white disulfonate has been added there is no further reduction of the ferricyanide. This is conclusive evidence that indigo disulfonate oxidizes all of the cobaltous cysteine to the brown cobaltic cysteine complex.

Oxidation of Cobaltous Cysteine with Oxygen and Hydrogen Peroxide

Eight solutions were prepared, all of which contained 20 cc. of 0.01 N cysteine and 10 cc. of 0.01 M cobalt sulfate. 0, 1, 2, 3, 4, 5, 6, and 7 cc. of 0.01 N hydrogen peroxide were added to the cells respectively. The cobaltous cysteine which still remained in solution after the addition of hydrogen peroxide, was determined by titration with indigo disulfonate. It was then found that the hydrogen peroxide had oxidized less than its equivalent of cobaltous cysteine to the brown cobaltic complex (Table I).

It is therefore evident that oxidation of cobaltous cysteine with hydrogen peroxide occurs simultaneously in two ways. One leads to the formation of the brown cobaltic cysteine complex. Quantitative evidence will be submitted to show that the other reaction is the conversion of cobaltous cysteine into cystine, and a cobaltous salt. The cystine was determined by a modification of the Folin-Looney method (5). The cobaltic cysteine complex has no effect on the uric acid reagent and the color due to the complex did not interfere as the comparisons were made in a Bürker colorimeter. After the cobaltous cysteine had been completely oxidized with oxygen, or hydrogen peroxide, and the cell had again been deoxygenated, the presence of a cobaltous salt was shown by the addition of more cysteine. The reduction of indigo disulfonate by the solution clearly showed the presence of a cobaltous salt which had formed cobaltous cysteine with the added cysteine.

The conditions under which the oxidation is carried out modify the quantitative relationships between the two products, the

cobaltic cysteine complex and cystine. If a solution of cobaltous cysteine is added to a solution of phosphate buffer pH 7.4 which contains hydrogen peroxide the amount of cobaltic complex which is formed may be only 44 per cent of the amount formed if the peroxide is added to the cobaltous cysteine. The amount of cystine formed is correspondingly increased. If cobaltous cysteine is added drop by drop to phosphate buffer pH 7.4, through which a rapid current of air is passed, less of the cobaltic complex and more cystine are formed than when the order of addition is reversed.

TABLE I

Relation between Volumes of Hydrogen Peroxide Reduced and Cobaltous Cysteine Oxidized

0.01 N hydrogen peroxide added	0.01 N indigo disulfonate required	Volume of 0.01 N cobaltous cysteine oxidized by peroxide
cc.	*cc.*	*cc.*
0	10.0	0.0
1	9.0	1.0
2	8.5	1.5
3	7.5	2.5
4	6.5	3.5
5	6.0	4.0
6	5.0	5.0
7	4.5	5.5

The difference between the volume of oxidant used and cobaltous cysteine oxidized is a measure of the cobaltous cysteine which was not oxidized to the cobaltic complex.

Under optimal conditions for the oxidation to the cobaltic complex, hydrogen peroxide and air both produce the same amount of cobaltic complex from a given amount of cobaltous cysteine.

Reaction between Cobaltous Cysteine and Ferrocyanide

Titration of a phosphate buffer solution of cysteine pH 7.4 with ferricyanide in the presence of varying amounts of cobalt shows that the concentration of cobalt has a great effect until the molar concentration is two-thirds that of the thiol group. This is in excess of the amount required to form cobaltous cysteine, and indicates that cobalt has some action other than the formation of cobaltous cysteine (Fig. 3).

The quantitative relationship is explained by the effect of the addition of ferrocyanide to a solution of cobaltous cysteine. For
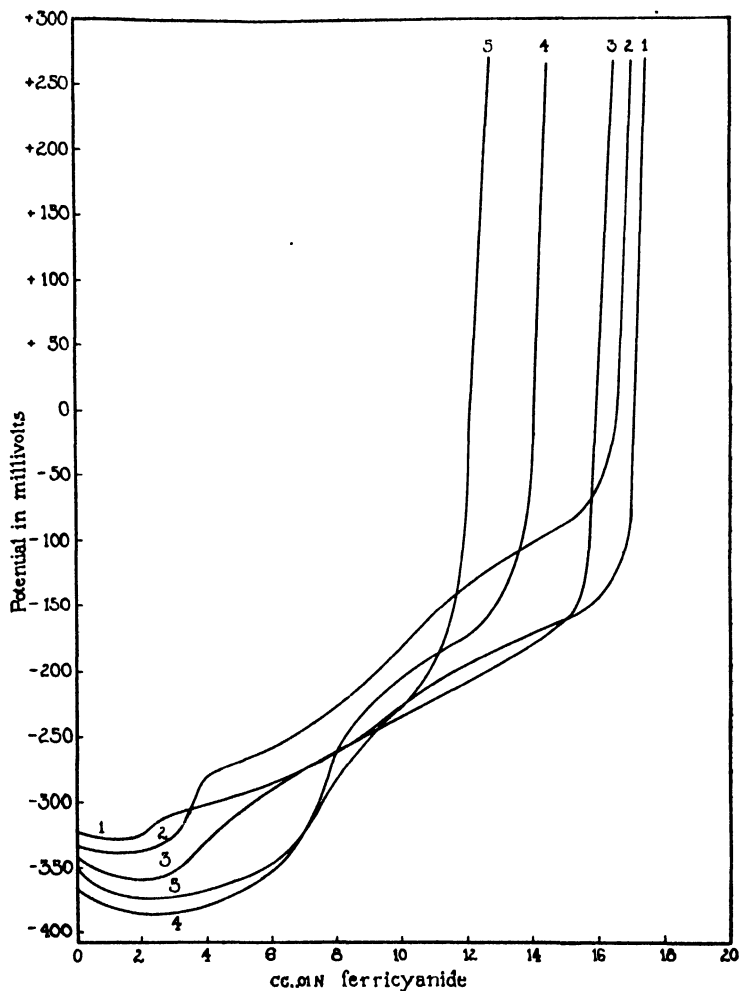
FIG. 3. Oxidation of cysteine with potassium ferricyanide in the presence of varying amounts of cobalt. Each of the solutions represented by the curves contained 2×10^{-4} mols of cysteine and cobalt sulfate in the following amounts 0.25, 0.5, 1.0, 2.0, and 3.0×10^{-4} mols.

each volume of a 0.01 M solution of ferrocyanide added an equivalent volume of 0.01 N indigo disulfonate is no longer required for

the oxidation of the cobaltous cysteine (Fig. 4). Ferrocyanide decomposes cobaltous cysteine with formation of cobalt ferrocyanide and cysteine.

The cobalt which is present in excess of the amount required to form cobaltous cysteine reacts with the ferrocyanide which is formed during the titration and prevents decomposition of the cobaltous cysteine.
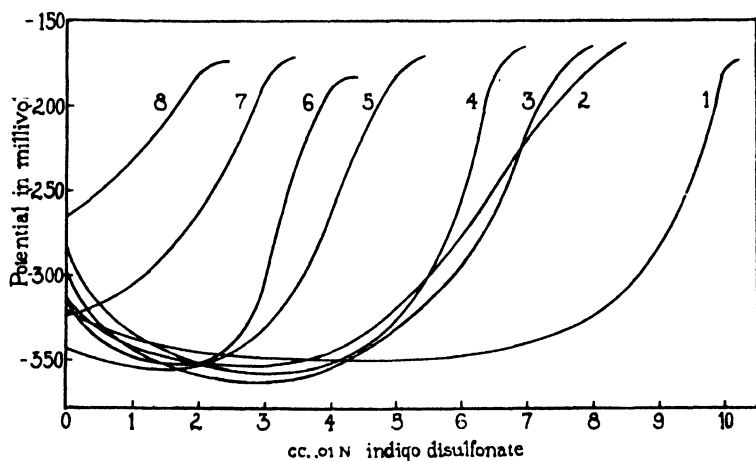
FIG. 4. The influence of ferrocyanide on the oxidation of cobaltous cysteine with indigo disulfonate. Each of the solutions represented by the curves contained 2×10^{-4} mols of cysteine and 1×10^{-4} mols of cobalt sulfate. To the solutions potassium ferrocyanide in the following amounts was added: 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7×10^{-4} mols. The cobaltous cysteine in solution was titrated with 0.01 N indigo disulfonate.

Sodium cyanide can also decompose cobaltous cysteine with the removal of cobalt ion from the solution and the liberation of cysteine (Fig. 5).

Formaldehyde and many other substances will bring about the decomposition of cobaltous cysteine and prevent its conversion to the cobaltic complex.

Although the presence of cobalt in excess prevents decomposition of cobaltous cysteine with ferrocyanide there is a limit to the protection afforded. It is impossible to secure a quantitative titration which represents only the oxidation of the cobaltous cysteine

to the brown cobaltic complex.   Even in the presence of a large excess of cobalt one-third of the cobaltous cysteine is oxidized to cystine by ferricyanide.
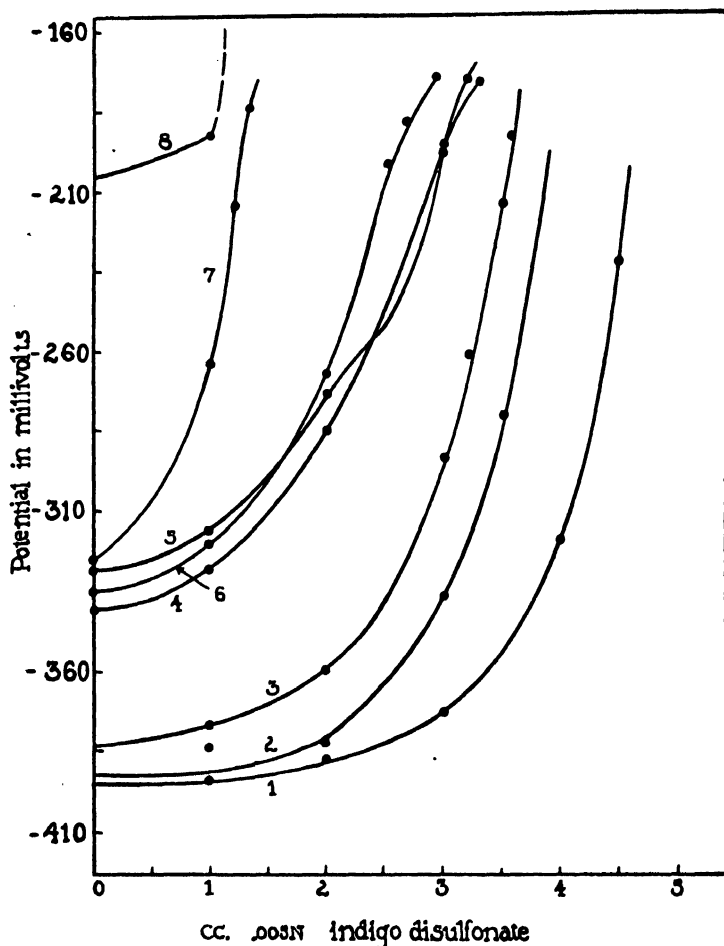
FIG. 5. The influence of sodium cyanide on the oxidation of cobaltous cysteine with indigo disulfonate.   Each of the solutions represented by the curves contained 2×10^{-4} mols of cysteine and 0.25×10^{-4} mols of cobalt sulfate.   Sodium cyanide was added to the cells in the following amounts respectively: 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4×10^{-4} mols.   The cobaltous cysteine was titrated with indigo disulfonate.

Significance of the Oxidation of Cobaltous Cysteine with Ferricyanide

In the presence of an excess of cobalt the total amount of ferricyanide required is a constant percentage of the concentration of the cysteine. Therefore, the amount of cobaltous cysteine which is oxidized to cystine must be a constant percentage of the total cysteine. As found by Michaelis and coworkers the total ferricyanide required is two-thirds of the concentration of the cysteine. Since the cobaltous cysteine is half the concentration of the cysteine, the amount of oxidant required is four-thirds the concentration of the cobaltous cysteine. Cobaltous cysteine requires 1 equivalent of an oxidant to form the complex and 2 equivalents to form cystine. With these quantities known it is possible to calculate how much of the cobaltous cysteine is oxidized to the brown cobaltic cysteine complex. Let x be the fraction of 1 mol of cobaltous cysteine which is converted to the brown complex. Then $(1 - x)$ is the fraction of cobaltous cysteine oxidized to cystine. Since four-thirds of an equivalent of oxidant is required for 1 mol of cobaltous cysteine, the number of equivalents of an oxidant required for oxidation of cobaltous cysteine in terms of the mols of cobaltous cysteine are $x + 2 (1 - x) = \frac{4}{3}$. From this x is found to be two-thirds of the cobaltous cysteine.

Expressed in terms of the oxidant, 10 cc. of 0.01 M cobaltous cysteine would require 6.7 cc. of 0.01 N ferricyanide to form the brown complex and 2×3.3 or 6.6 cc. to form the cystine. The total amount would be 13.3 cc. which is the volume found by titration.

Expressed in terms of the two reactions, for every 2 molecules of cobaltous cysteine which are oxidized to the cobaltic complex, 1 molecule of cobaltous cysteine is oxidized to cystine. In terms of cysteine, when 2 molecules of cysteine are converted into the complex another molecule of cysteine is oxidized to furnish a half molecule of cystine.

Oxidation of cobaltous cysteine to the cobaltic complex and of cysteine to cystine are competing reactions but titration of a solution of cobaltous cysteine with indigo disulfonate after the addition of varying amounts of ferricyanide shows that all cobaltous cysteine has been removed from solution when two-thirds of it has

been oxidized to the complex. Cysteine is not oxidized to cystine until all cobaltous cysteine has been acted on. The two reactions are sharply separated. The reason for this appears to be the specific nature of the oxidizing agent. The probable mechanism is discussed on p. 454.

Reaction between Cysteine and Phenolindophenol

In the absence of sufficient cobalt, ferricyanide brings about a side reaction which modifies the course of the oxidation of cobaltous cysteine and with phenolindophenol still another side reaction has been found which also modifies the course of the oxidation.

In order to show the effect of cobalt on the oxidation of cysteine eight solutions all of which contained the same amount of cysteine and increasing amounts of cobalt were titrated with dibromophenolindophenol (Fig. 6). Until the concentration of the cobalt was equal to half that of the cysteine the amount of the dye which was required was determined by the concentration of the cobalt (Curves 1 to 3). As soon as the cobalt was present in amount equal to half the concentration of the cysteine, the addition of more cobalt did not appreciably affect the volume of the dye required. (Curves 4 to 8).

The side reaction which occurs between cobaltous cysteine and phenolindophenol does not remove cobalt ion from solution. It is between the thiol group of cysteine and the quinone group of phenolindophenol. Kendall and Mason (9) have shown that if quinone is added to a solution of cysteine, the cysteine is not oxidized to cystine but the thiol group adds to the quinone probably similarly to the addition of aromatic thiol groups to quinone
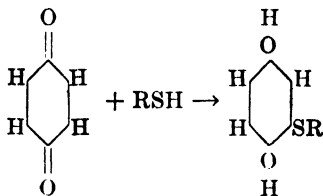
For each molecule of cysteine 1 molecule of quinone, that is, 2 equivalents of oxidant are required. If the solution is acidified and extracted with butyl alcohol, cystine is not found in the solu-

tion.   The sulfur originally present as the thiol group is quanti-
tatively extracted in combination with the quinone.   The product
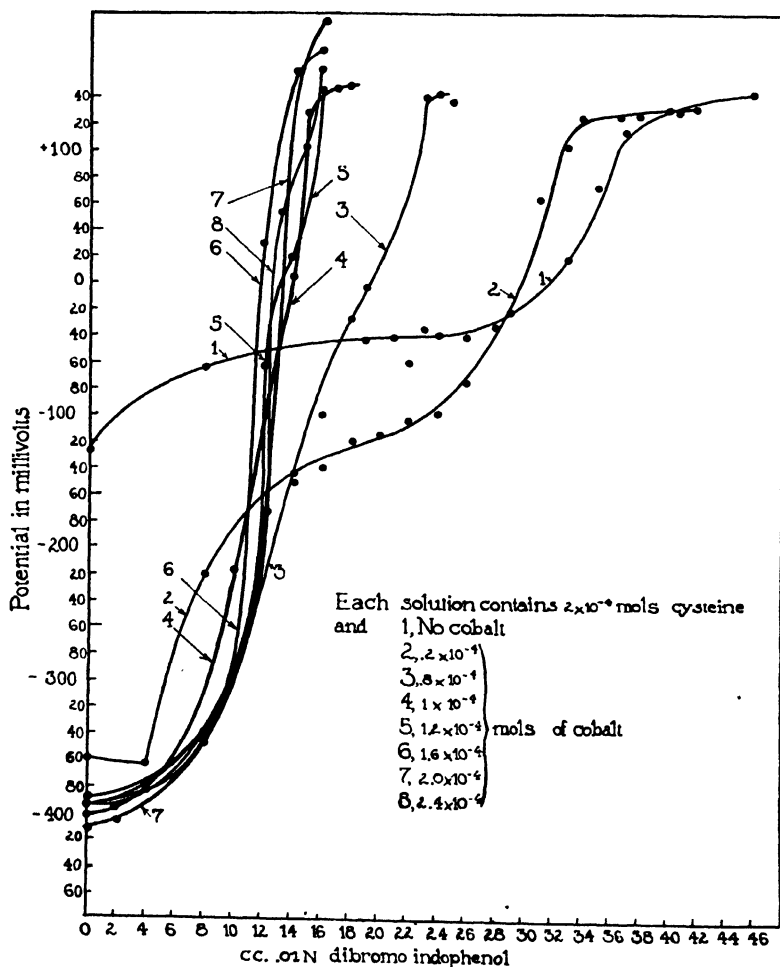
FIG. 6. The oxidation of cysteine in the presence of varying amounts of
cobalt with dibromophenolindophenol.   In the absence of cobalt each mol
of cysteine reacts with 2 mols of the dye.   In the presence of cobalt the
amount of oxidant is reduced to the amount of ferricyanide required.

which results from the addition of cysteine to quinone exists in two stages of oxidation corresponding to quinol and quinone.

Cysteine reacts with phenolindophenol and with dibromophenolindophenol as it reacts with quinone. An addition product is formed through the thiol group of 1 molecule of cysteine and the quinone group of 1 molecule of the dye. This compound exists in two stages of oxidation, the reduced form is colorless and the oxidized is blue. The reduced form is oxidized to its quinone by phenolindophenol.

TABLE II

Relation between Volumes of Dibromophenolindophenol Reduced and Cobaltous Cysteine Oxidized

0.01 N dibromophenolindophenol added	0.01 N indigo disulfonate required	Volume of 0.01 N cobaltous cysteine oxidized by dibromophenolindophenol dye
cc.	*cc.*	*cc.*
0	10.0	0.0
1	9.0	1.0
2	8.0	2.0
3	7.0	3.0
4	6.0	4.0
5	5.5	4.5
6	4.5	5.5
7	3.5	6.5
8	3.5	6.5
9	2.0	8.0
10	1.0	9.0

The difference between the volume of dibromophenolindophenol and cobaltous cysteine oxidized is a measure of the cobaltous cysteine that was not oxidized to the cobaltic cysteine complex.

Quantitative evidence that a side reaction occurs with phenolindophenol was secured as follows: Ten electrode cells were prepared, all of which contained 20 cc. of 0.01 N cysteine and 10 cc. of 0.01 M cobalt sulfate. To these solutions 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 cc. of 0.01 N dibromophenolindophenol were added respectively. The cobaltous cysteine which still remained after the addition of dibromophenolindophenol was then determined with indigo disulfonate. The amounts of indigo disulfonate required to complete the oxidation of the cobaltous cysteine showed that the dibromophenolindophenol had oxidized less than its equiv-

alent of cobaltous cysteine to the brown complex.   The cobaltous
cysteine had been oxidized in two ways, one of the end-products
was the cobaltic complex but no cystine was formed.   The cysteine
which had not been converted into the cobaltic complex had com-
bined with the dye (Table II).

The oxidation of cobaltous cysteine to the cobaltic complex and
the addition to the dye must proceed simultaneously.   In this
respect the oxidation with dibromophenolindophenol is quite
different from oxidation with ferricyanide.   With ferricyanide
even in the presence of excess cobalt all of the cobaltous cysteine
is removed from the solution by the addition of half of the total
ferricyanide required.   10 per cent of the total cobaltous cysteine
is present even after dibromophenolindophenol has been added
in amount sufficient to oxidize all of the cobaltous cysteine to the
brown complex.

The order of addition of cobaltous cysteine and phenolindo-
phenol markedly changed the percentage of the cobaltous cysteine
which was converted into the cobaltic complex.

Form of the Potentiometric Oxidation Curve with Ferricyanide and
with Phenolindophenol

When Michaelis and Barron (13) carried out the oxidation of
cobaltous cysteine with ferricyanide and with phenolindophenol,
two different types of curves were obtained.   It appeared probable
that:

"The titration detects, however, something new which cannot
be recognized by the gas analytical method."

"As far as we can see, the case of cobaltous cysteine is the first
one in which the difference in these two kinds of oxidants is mani-
fested.   We may speak of ferricyanide as a *single step oxidant*,
of indophenol as a *double step oxidant*, and of cobaltous cysteine as a
two single step reductant."

Cannan and Richardson (1) have objected to this explanation
for the shape of the curve and suggested that possibly ferricyanide
was not a satisfactory oxidant.   The quantitative relationships
shown in the curve, Fig. 4, in the paper by Michaelis and Barron
(13) are explained by the results that have been given in this paper.

The ratio of cysteine to cobalt was 3 : 2.   This is sufficient cobalt
to form the complex and prevent decomposition of the cobaltous

cysteine with the ferrocyanide. The complete removal of cobaltous cysteine from the solution is indicated by the sharp break in the curve which occurs at a point between 0.48 and 0.52 cc. on the abscissa. Oxidation with ferricyanide results in the complete removal of cobaltous cysteine with its high reduction intensity in the first half, and oxidation of the thiol group of cysteine in the second half of the titration. These two steps fully explain the form of the curve obtained with this oxidant. The cobaltous cysteine was all removed by a volume of ferricyanide just equal to two-thirds the concentration of the cobaltous cysteine and to half the total oxidant required. The quantitative relationships therefore agree with those which have been given.

Since cobaltous cysteine requires but 1 equivalent of an oxidant to form the brown cobaltic cysteine complex, the explanation of the shape of the curve suggested by Michaelis and Barron (13) cannot be correct.

In sharp contrast to oxidation with ferricyanide the curve which represents titration with phenolindophenol is a gradual decrease in potential with each addition of the dye. This curve is in keeping with the fact that cobaltous cysteine is present until the end of the titration. The two reactions, oxidation to the cobaltic complex and addition to the dye, occur simultaneously and throughout the entire titration.

Oxidation of Cobaltous Cysteine with Cystine

Michaelis and Barron (13) showed that cobaltous cysteine can be oxidized to the brown cobaltic complex with cystine. In their experiments, however, cystine always produced less of the brown complex than was formed by molecular oxygen or other oxidizing agents from the same amount of cobaltous cysteine. We have confirmed the observation that cobaltous cysteine is oxidized with cystine but we have found that if sufficient cystine is present and sufficient time is given the oxidation of cobaltous cysteine is complete.

Previous work by many investigators has shown that cysteine and cystine do not form a dynamic equilibrium. The reduction of the disulfide grouping requires the most powerful reducing agents. By the use of chromous chloride Preisler (16) has reduced cystine at a measurable velocity although this was carried out in acid solu-

tion. The inertia of the disulfide grouping appears to be overcome in the presence of cobaltous cysteine. Both the velocity and extent of oxidation of cobaltous cysteine with the disulfide grouping are determined by the ratio of cystine to cysteine (Figs. 7 to 9).

Cobalt Salts and Glutathione

Cobalt and glutathione do not form a cobaltic complex. Cobalt and cystine do not react but if glutathione is added to such a solu-
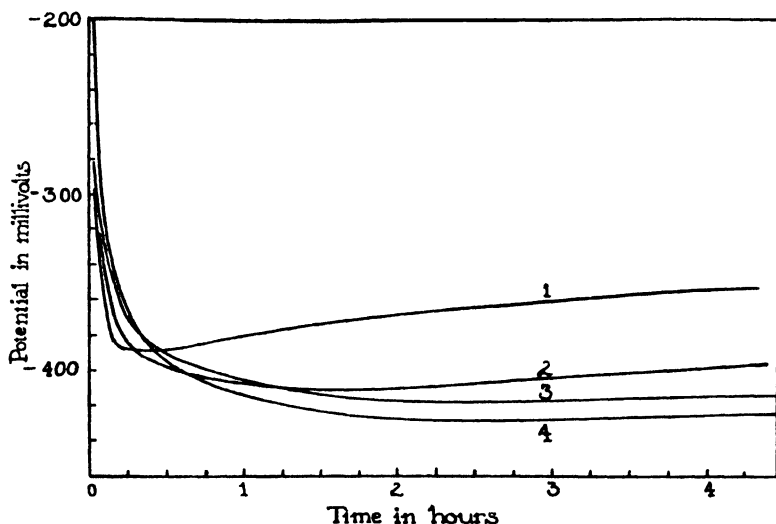
Fig. 7. Oxidation of cobaltous cysteine with small amounts of cystine. Each of the solutions represented by the curves contained 1×10^{-4} mols of cystine and 1×10^{-4} mols of cobalt sulfate. The following amounts of cysteine were added to Cells 1, 2, 3, and 4 respectively, 0.4, 2, 5.6, and 8×10^{-4} mols.

tion, the thiol group of glutathione will react with cystine with the formation of cysteine which will then form cobaltous cysteine and be oxidized to the brown cobaltic cysteine complex by the excess cystine. Cobaltous cysteine is also oxidized with the disulfide group of oxidized glutathione as it is with cystine.

Oxidation of Cobaltous Cysteine with Sodium Hydrosulfite and Sulfite

The remarkable activity of cobaltous cysteine and its great tendency to form a cobaltic cysteine complex is well illustrated by the formation of a complex with two substances which are under
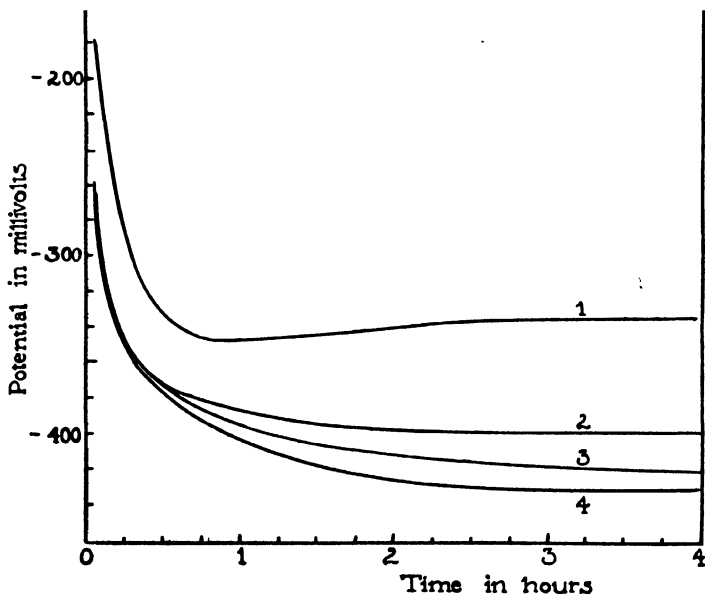
FIG. 8. The oxidation of cobaltous cysteine in the presence of large amounts of cystine. Each of the solutions represented by the curves contained 4×10^{-4} mols of cystine and 1×10^{-4} mols of cobalt sulfate. The following amounts of cysteine were added to Cells 1, 2, 3, and 4 respectively, 1.6, 8, 22.4, and 32×10^{-4} mols. The more positive potentials shown in this chart compared to Fig. 7 show the influence of the increased amount of cystine.

most conditions employed as reducing agents. Sodium hydrosulfite added to a solution of cobaltous cysteine brings about an almost instantaneous conversion to a brown cobaltic cysteine complex. At the same time the intense reduction potential due to the presence of the hydrosulfite is rapidly changed to a much more positive value and the potential finally reached is the same as that

of a solution of cobaltous cysteine treated with a more powerful oxi-
dant. When the color of the solution was compared in a spectro-
photometer it was found to be the same as the color produced from
cobaltous cysteine with oxidants. The solution, however, was
more opaque. Since the brown compound formed from cobaltous
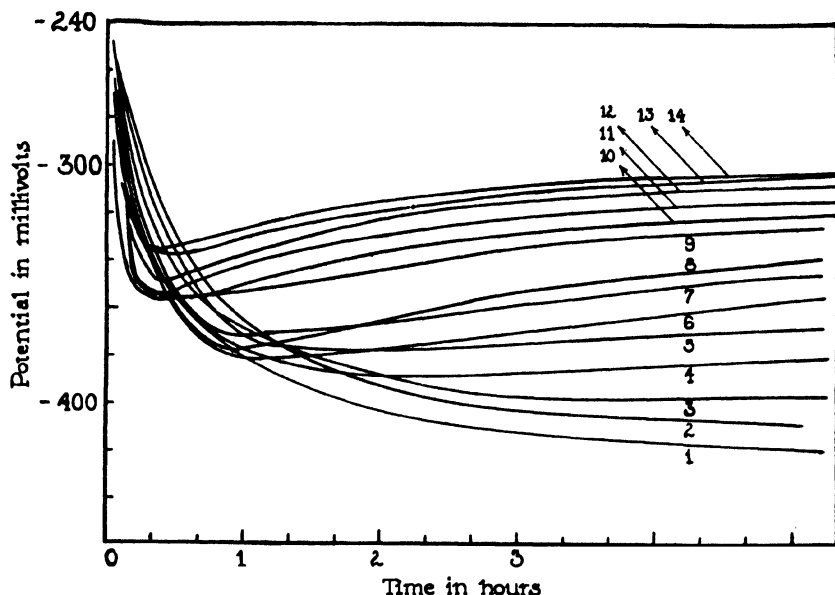
FIG. 9. The equilibrium between cystine and cysteine in the oxidation
of cobaltous cysteine. Each of the solutions represented by the curves
contained 2×10^{-4} mols of cysteine and 1×10^{-4} mols of cobalt. The fol-
lowing amounts of cystine were added to the electrode cells respectively:
0.0, 0.4, 0.8, 1.2, 2, 2.8, 3.6, 4.4, 4.8, 5.6, 8, 6.4, 7.2, and 8.8×10^{-4} mols. The
curves show that the development of the high reduction potential from
cobaltous cysteine is prevented by the presence of large concentrations of
cystine. Both the velocity and the completeness of the oxidation are
determined by the ratio of cystine to cysteine.

cysteine with hydrosulfite has not been analyzed it is impossible
to say that it is identical with the brown cobaltic complex formed
with oxygen or other oxidant. In a number of chemical properties
and in color, however, it appears to be the same compound.

Sodium sulfite will also convert cobaltous cysteine into the cobal-
tic complex but with this reagent the solution is not more opaque

than a solution of cobaltic cysteine complex which has been prepared with molecular oxygen. Sodium sulfite, cystine, and oxidized glutathione are the slowest of the oxidants. It requires many hours to reach equilibrium. After 24 hours sodium sulfite oxidized 90 per cent of the cobaltous cysteine. The intensity of color was less than that produced with cystine and the solution reduced 10 per cent of the total theoretical amount of indigo disulfonate.

Sodium thiosulfate did not cause the formation of any of the cobaltic complex.

Hydrogen sulfide precipitates cobalt sulfide from a solution of cobaltous cysteine, but will not precipitate cobalt sulfide from a solution of the cobaltic cysteine complex at a pH of 7.4 although the complex is slowly decomposed with hydrogen sulfide in the presence of hydrochloric acid.

Amount of Brown Cobaltic Cysteine Complex Formed from Cobaltous Cysteine by Oxidants

Michaelis and Barron (13) utilized the color produced in solution as a criterion of the amount of cobaltous cysteine which had been oxidized to the brown complex. This would appear to give an accurate determination since no other colored compound is formed by oxidation of cobaltous cysteine, and it has made possible a quantitative measure of the complex except with three of the oxidants.

With indigo disulfonate it is impossible to secure more than a rough approximation. This is because of the color of indigo disulfonate either in the oxidized or reduced form. If the dye is kept in the reduced form by the addition of hydrosulfite the depth of color rapidly increases. If the oxidized form is removed with activated charcoal some of the brown cobaltic complex is also removed from the solution. An approximate determination of the color can be made by direct comparison of the solution in the electrode cell with a solution of the complex prepared with another oxidant.

It has been impossible to determine the amount of cobaltic complex formed with ferricyanide. In all of the solutions the precipitate of cobaltous ferrocyanide produced a turbidity which appeared to adsorb the complex. Complete removal of ferrocyanide with excess of cobalt also removed some of the complex.

It is impossible to determine the amount of cobalt complex produced with hydrosulfite. The solution is of the same color but is too opaque for comparison.

The color produced by the oxidation of cobaltous cysteine with air has been taken as the standard. The slow addition of hydrogen peroxide to a solution of cobaltous cysteine developed the same intensity of color as the standard. With dibromophenolindophenol the dye was removed with butyl alcohol and then the color was 113 per cent of the standard. With cystine the color was 133 per cent of the standard and with sodium sulfite 111 per cent. The color produced by indigo disulfonate was a little greater than that produced by dibromophenolindophenol. See Tables III and IV.

By determination of the intensity of color, therefore, the conclusions which were drawn from the quantitative determination of cobaltous cysteine through titration with indigo disulfonate have been confirmed.

Composition of the Brown Cobaltic Cysteine Complex

It is impossible to assign a structure to the brown complex. It appears to contain 1 atom of cobalt and 2 molecules of cysteine. That the amino group of cysteine is involved in the formation of the complex is indicated by the fact that glutathione will not form a complex with cobalt. If, however, the glutamyl grouping is hydrolyzed from the tripeptide, the cysteyl glycine (10, 12) will form a complex with cobalt.

The carboxyl group of cysteine appears to be involved in the complex. The ethyl ester of cysteine will react with cobalt sulfate and the resulting product can be oxidized with indigo disulfonate. However, the olive green described by Michaelis and Barron (13) which is characteristic of cobaltous cysteine is not formed with the ester. The solution is water white. Although a brown color is produced by oxidation of cobaltous cysteine ester, it is insoluble and separates as a voluminous product in long streamers which adhere to the platinum electrode and side of the electrode vessel.

It therefore appears probable that the thiol group, the amino group, and the carboxyl group are all involved in the formation of the brown cobaltic cysteine complex and that the six coordination valencies of the cobaltic complex are satisfied by these three groups in the 2 molecules of cysteine which are combined with the cobalt.

Possible Reaction Mechanism of the Oxidation of Cobaltous Cysteine

It has been shown that when cobaltous cysteine is oxidized with oxygen, hydrogen peroxide, ferricyanide, and phenolindophenol, the end-products are a brown cobaltic cysteine complex and either cystine or a cysteine dye addition product. The quantitative relation between these end-products suggests that a definite reaction mechanism is involved which is identical with all four oxidizing agents. There are seven observations which indicate the nature of the reaction.

1. The reduction potential of cobaltous cysteine is high but its full value is not registered on the platinum electrode for many hours. In the absence of an oxidant the potential may not reach its highest value even after 6 hours. If, however, a small amount of an oxidant (1 cc. of 0.01 N solution) is added the reduction potential immediately increases to its maximum. The reaction that causes the development of a high reduction potential is catalyzed by the addition of an oxidant (Fig. 10).

2. The reduction potential of cobaltous cysteine is much higher than that of cysteine. The higher value indicates an increase in the intensity of the thiol group as indigo disulfonate in the presence of excess cysteine can form traces of cystine.

3. As shown by Michaelis and Barron (13) the maximal reduction potential of a solution of cobaltous cysteine is almost equal to that of a hydrogen electrode in the same hydrogen ion concentration. However, the addition of indigo disulfonate suppresses the reduction potential for an appreciable interval. The reduction of the dye involves a time reaction. This is also true with phenolindophenol and with hydrogen peroxide. This effect is more marked near the end of a titration of cobaltous cysteine.

4. The conversion of cobaltous cysteine into the cobaltic complex is not a straight forward oxidation of the cobalt alone. That an intermediate oxidation product is involved is indicated by the alteration in the proportion of complex and of cystine caused by a reversal in the order of addition of oxidant and the solution of cobaltous cysteine.

5. Under the optimal conditions for the oxidation of cobaltous cysteine to the cobaltic complex with ferricyanide, one-third of the
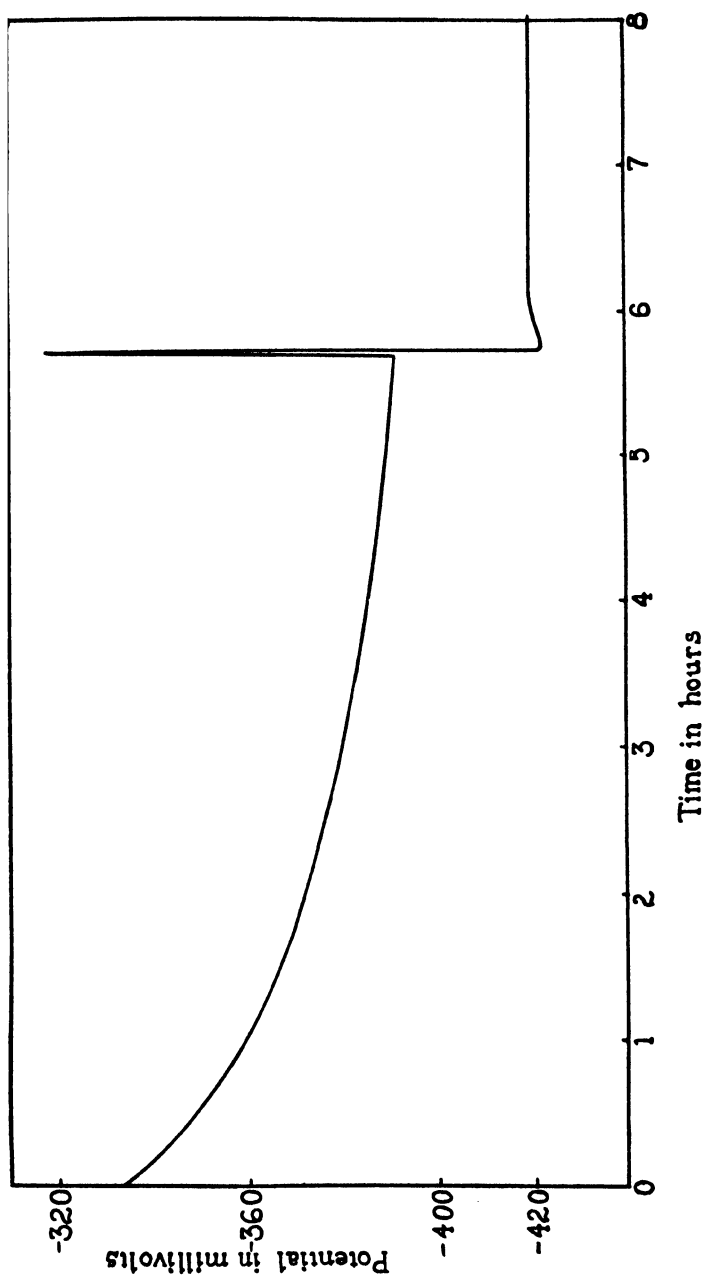
Fig. 10. The reduction potential of a solution of cobaltous cysteine. The solution contained 2×10^{-4} mols of cysteine and 1×10^{-4} mols of cobalt sulfate. The curve shows a slow increase in the reduction potential for 5 hours. The sharp break in the curve was caused by the addition of 1 cc. of 0.01 N hydrogen peroxide. The same effect was obtained with solutions of the same strength of all the other oxidants.

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total cysteine is oxidized to cystine, and two-thirds to the cobaltic complex. The same amount of cobaltic complex is formed if cobaltous cysteine is added to dibromophenolindophenol.

6. The oxidation of cobalt sulfate to the cobaltic state will not occur with any of the oxidants at this pH unless the thiol group is present.

7. Oxidation of cobaltous cysteine cannot be merely a change in valence of the cobalt as it occurs in a complex similar to the change from cobaltocyanide to cobalticyanide. Cobaltous cysteine does not form a stable complex. Hydrogen sulfide will precipitate cobalt and ferrocyanide and cyanide ion will decompose it. The probable course of the oxidation is the production of a cobaltic salt which is ionized.

These seven experimentally established facts may all be related through a common intermediate oxidation product. The accompanying hypothesis is based on the reactions which have been described.
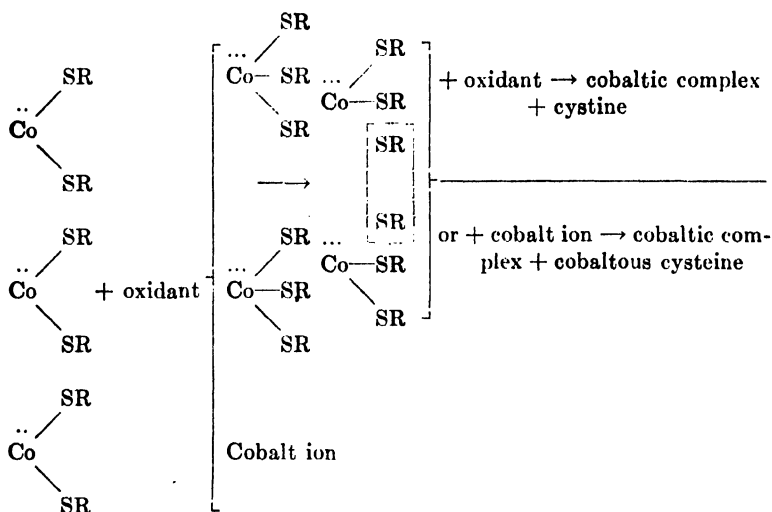
The first step is the production of cobaltic ion in the form of cobaltic tricysteine. Although the first step is dependent on the presence of the thiol group, cobaltic tricysteine is less stable than the cobaltic dicysteine complex into which the cobaltic tricysteine is converted with liberation of a negatively charged ion −SR.

In the presence of an oxidant the negatively charged $-SR$ group is converted into cystine. In the absence of an oxidant that can oxidize it the $-SR$ group remains in solution as cysteine or recombines with cobalt.
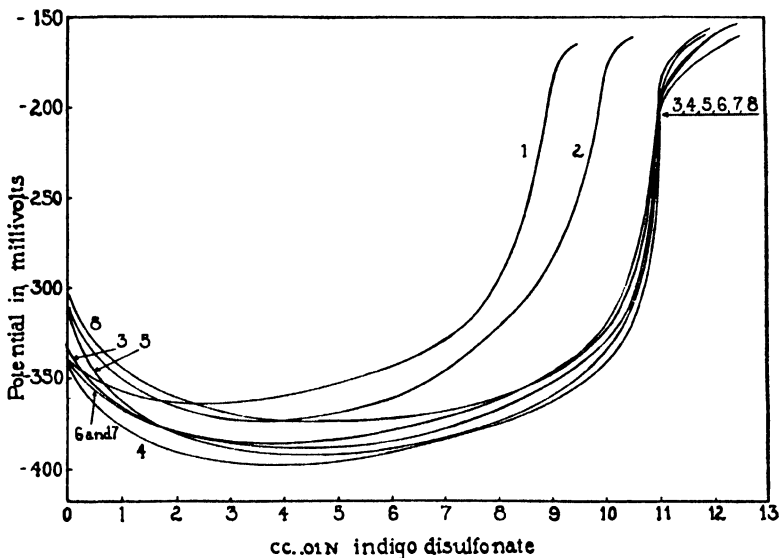
FIG. 11. Oxidation of cobaltous cysteine in the presence of an excess of cysteine. Each of the solutions represented by the curves contained 1 × 10^{-4} mols of cobalt sulfate and cysteine in the following amounts: 1.8, 2.4, 3.0, 3.6, 4.2, 4.8, 5.4, and 6.0 × 10^{-4} mols. When less cysteine than twice the molar concentration of cobalt was present the amount of indigo disulfonate required was equal to half the concentration of the cysteine. In the presence of cysteine, which was equal to more than twice the molar concentration of the cobalt, approximately 10 per cent more of the oxidant was required. The amount of oxidant required soon reached a limit and was not proportional to the increase in concentration of cysteine. The increase in volume of oxidant was due to oxidation of cysteine to cystine. Cobalt ion is necessary for this reaction, oxidation to the disulfide does not occur unless cysteine is present in excess.

This hypothesis for the reaction mechanism agrees with the observation of the reduction potential of cobaltous cysteine before and after the addition of an oxidant. The conversion of the tricysteine cobaltic intermediate into the stable cobaltic cysteine complex with liberation of the negatively charged $-SR$ ion would explain the observed intensity.

Indigo disulfonate does not oxidize cobaltous cysteine to cystine unless cysteine is present in excess. The oxidation of a small percentage of the cysteine to cystine is evidence that the $-SR$ group has been increased in its activity above the activity of the thiol group of cysteine which cannot be oxidized to cystine with indigo disulfonate (Fig. 11).

The conversion of cobaltic tricysteine to the cobaltic complex involves a time reaction. Therefore oxidants would bring about the conversion of variable proportions of cobaltous cysteine to the complex and to cystine depending on the nature of the oxidizing agent and the conditions under which the oxidation was carried out.

Ferricyanide converts one-third of the cobaltous cysteine to cystine. The oxidation of the $-SR$ group as it is liberated from the tricysteine cobaltic salt in the presence of phenolindophenol would result in its addition to the quinone group of the dye instead of the formation of cystine.

Quantitative Relations of the Oxidants in the Oxidation of Cobaltous Cysteine

With ferricyanide cobaltous cysteine is converted into the two products, cobaltic cysteine complex and cystine. With dibromophenolindophenol no cystine is formed, and still the volume of dye required is the same as the equivalent solution of ferricyanide. The relation between the amount of dibromophenolindophenol reduced and the amount of complex formed can be clearly established. When cobaltous cysteine is oxidized to the complex 2 molecules of cysteine are converted into the cobalt complex by 1 equivalent of the dye. When cysteine combines with the quinone group of the dye each molecule of cysteine reduces 2 equivalents of the dye. The ratio of the volumes of oxidant required are as 1:4. The equation for the oxidation in terms of cobaltous cysteine and the dye may be written

$$x + 4\,(1 - x) = \frac{\text{cc. of 0.01 N dye}}{\text{cc. of 0.01 M cobaltous cysteine}}$$

in which x is the fraction of cobaltous cysteine converted to the complex. 13.3 cc. of 0.01 N dye are required for 10 cc. of 0.01 M cobaltous cysteine. x is therefore 89 per cent.

The amounts of brown cobaltic complex and of cystine formed by titration with the various oxidants are close to the theoretical except when hydrogen peroxide is used. With oxygen and hydrogen peroxide the average amount of cystine formed was 23 per cent of the total cysteine. This would indicate that 77 per cent of the cobaltous cysteine had been converted into the complex. For 10 cc. of 0.01 M cobaltous cysteine the total titration would be 7.7 + 2 × (2.3) or 12.3 cc. The amount of hydrogen peroxide reduced was 13.3 cc. Although the discrepancy between the calculated and actual amount required is not great, the difference is well beyond the experimental error. This suggests that the end-products are not only the cobaltic complex and cystine.

The most probable explanation is that the highly reactive −SR group may be oxidized beyond the disulfide stage with the formation of small amounts of cysteic acid. The oxidation of traces of cysteine to cysteic acid would explain the discrepancy between the amount of cystine and cobalt complex formed and the volume of oxidant required. 6 equivalents of oxidant are required to convert 1 molecule of cysteine to cysteic acid: this is 12 times the amount required to convert cysteine into the cobaltic cysteine complex.

The quantitative relationships can be summarized as follows, in each case the volume of cobaltous cysteine is 10 cc. of 0.01 M solution, and the oxidant is a 0.01 N solution. With indigo disulfonate 10 cc. of the cobaltous cysteine would be oxidized to the brown cobaltic complex; no cystine would be formed. The volume of oxidant required is 10 cc. With ferricyanide in the presence of sufficient cobalt to react with the ferrocyanide, 6.7 cc. of the cobaltous cysteine would be oxidized to the complex and 3.3 cc. to cystine. Total volume of oxidant 6.7 + 2 × (3.3) = 13.3 cc. With dibromophenolindophenol 8.9 cc. of the cobaltous cysteine would be oxidized to the complex and 1.1 cc. would combine with the dye. Total oxidant required 8.9 + 4 × (1.1) = 13.3.

With hydrogen peroxide and with air 7.6 cc. of the cobaltous cysteine would be converted into the cobaltic complex, 2.3 cc. into cystine, and 0.1 cc. into cysteic acid. Total volume required 7.6 + 2 × (2.3) + 12 × (0.10) = 13.4.

The same amount of the three oxidants, ferricyanide, hydrogen peroxide, and phenolindophenol, is required but this is only coincidence. A different side reaction is involved with each oxidant.

The quantitative relations in the oxidation of cobaltous cystine with the different oxidants can be explained by variations in the relative velocities of four reactions: (1) oxidation of cobaltous cysteine to cobaltic tricysteine; (2) conversion of cobaltic tricysteine into the cobaltic dicysteine complex; (3) oxidation of the $-SR$ group to cystine or addition to the quinone group of a dye; (4) recombination of the $-SR$ group with cobalt ion.

The velocities of (1) and (3) are variable and depend on the oxidant used. The velocities of (2) and (4) are fixed for any given temperature and pH but (4) is influenced by the presence of anions, such as ferrocyanide. If (1) is rapid compared with (2), then (1) will take place completely before (3) can occur at all. If (4) is slow the amount of cobaltic complex formed approaches the minimal value, 67 per cent of the cobaltous cysteine. Both of these results are obtained when ferricyanide is added to cobaltous cysteine. If the velocity of (1) is rather small or if (1) and (2) are nearly equal, (1) and (3) will take place simultaneously. This result is obtained with phenolindophenol, hydrogen peroxide, and oxygen.

Variations in the per cent of cobaltous cysteine which follow reaction (3) are determined by changes in the relative velocities of reactions (3) and (4). Reaction (4) can be eliminated with dibromophenolindophenol by the simultaneous addition of cysteine and cobalt sulfate to the dye in phosphate buffer pH 7.4. Under these conditions only 67 per cent of the cobaltous cysteine is converted into the cobaltic complex. If the dye is added to cobaltous cysteine 89 per cent is converted to the cobaltic cysteine complex.

If the velocity of reaction (3) approaches zero, recombination of the $-SR$ group with cobalt ion will bring about the conversion of 100 per cent of the cobalt into the cobaltic complex. This result is obtained with indigo disulfonate, however, in the presence of an excess of cysteine, a small percentage of the $-SR$ group of the excess cysteine is converted into cystine.

From a biological standpoint, and in particular from the viewpoint of the action of iron on the oxidation of cysteine, the conversion of cobaltous cysteine into cystine may be considered the main reaction. The formation of the cobaltic cysteine complex under.

the condition of the experiment is an irreversible side reaction which removes both the cobalt and the cysteine from further action.

The essential features of the apparatus have been described (8, 11). Throughout the entire series of experiments the same buffer, Sorenson's (2) phosphate mixture, 80 cc. of secondary sodium phosphate $\frac{M}{15}$, and 20 cc. of primary potassium phosphate $\frac{M}{15}$ in a total volume of 100 cc. were used. The pH of the solution was checked with the hydrogen electrode and with the potential of added quinhydrone.

Electrodes—Bright platinum wire electrodes were used throughout. They were shown to agree with each other within 0.2 millivolt when compared with the same buffer in the presence of quinhydrone. A calomel half-cell in saturated potassium chloride was used and all readings are referred to the normal hydrogen electrode by subtracting 0.242 from the observed readings. The potential of the calomel electrode was checked with the hydrogen electrode in 0.05 M potassium acid phthalate. The value of -0.4804 volt was obtained which gives an indicated potential of -0.242 in reference to the normal hydrogen electrode. The oil thermostat was maintained at 30° ±0.2°.

Potassium Ferricyanide—This was purified according to the method of Folin (4) from commercial potassium ferricyanide.

Potassium Ferrocyanide—A commercial sample of this salt was used without recrystallization.

Cobalt Sulfate—The highest grade commercial sample of crystalline cobalt sulfate, $CoSO_4\ 2H_2O$, was used without further purification. It contained 97.7 per cent of the theoretical amount of cobalt and 97.4 per cent of the theoretical amount of sulfate.

Sodium salt of dibromophenolindophenol was prepared in this laboratory by the method of Möhlau (15).

Sodium salt of indigo disulfonate was purified from a commercial preparation by solution in water and precipitation with sodium acetate. This was repeated. The precipitate was then washed with water and dried to constant weight.

Cystine was prepared from human hair according to the method of Gortner and Hoffman (7). Iron was removed by precipitation with potassium ferricyanide. The cystine was recrystallized from solution and all traces of potassium ferricyanide were removed with water.

Cysteine Hydrochloride—Cystine was reduced with tin and hydrochloric acid. The tin was removed with hydrogen sulfide and the hydrochloride of cysteine was crystallized after the solution had been concentrated in a vacuum. The crystals were washed with acetone. The cysteine hydrochloride was titrated in 95 per cent alcohol with iodine and in phosphate buffer with potassium ferricyanide. The theoretical amount of each was required.

The ester of cysteine was prepared from cysteine by the use of absolute alcohol and dry hydrogen chloride. It was crystallized from absolute alcohol. M. p. 125°. Nitrogen by micro-Kjeldahl 7.48, calculated 7.55.

Glutathione was prepared by a method which has been described (10). It was recrystallized from water and the titration of its thiol group with iodine in alcohol and with potassium ferricyanide in phosphate buffer showed that it was a pure preparation of the tripeptide. The disulfide form was prepared by aeration of the barium salt in faintly alkaline solution. The purity of the sample was established by Dr. H. L. Mason according to results which will be published from this laboratory in the near future.

The electrode cells containing cysteine were prepared in the following manner. 0.320 gm. of cysteine hydrochloride were dissolved in 100 cc. of water. This was placed in a movable burette similar to the one originally described by Clark and Cohen (3) and the solution was deoxygenated. 2 cc. of 0.01 N sodium hydroxide and 100 cc. of phosphate buffer were placed in each of the eight electrode cells and deoxygenated. The alkali was just sufficient to react with the hydrochloride of cysteine so that the cysteine was present in a phosphate buffer pH 7.4. 10 cc. of the deoxygenated solution of cysteine hydrochloride were added to each of the electrode cells. If cobalt sulfate was used it was either added to the buffer in the cell before the cysteine, as a deoxygenated solution from a burette after the cysteine, or it was dissolved in the solution of cysteine hydrochloride. No reaction occurs between cysteine hydrochloride and cobalt sulfate at the pH of this solution. When

cobaltous cysteine was added to the oxidizing agent the mixture of cysteine and cobalt was brought to pH 7.4 by the addition of oxygen-free buffer and sodium hydroxide. This step was carried out in the absence of oxygen.

Cystine was dissolved with 2 equivalents of hydrochloric acid in a small volume of water and added to the buffer which contained sufficient alkali to neutralize the mineral acid.

Oxidation with Ferricyanide of Indigo White Disulfonate Produced by Reduction of Indigo Disulfonate with Cobaltous Cysteine—2 × 10^{-4} mols of cysteine and 1 × 10^{-4} mols of cobaltous sulfate were added to an electrode cell containing 100 cc. of buffer pH 7.4 and deoxygenated. The solution required 10 cc. of 0.01 N indigo disulfonate for complete oxidation of the cobaltous cysteine and an equal volume of 0.01 N ferricyanide was required to oxidize the indigo white disulfonate. The end-point was determined potentiometrically with each oxidant.

Evidence for Presence of Cobalt Ion in Solution after Oxidation of Cobaltous Cysteine with Various Oxidants—Eight electrode cells were prepared which contained 2 × 10^{-4} mols of cysteine hydrochloride, and 1 × 10^{-4} mols of cobalt sulfate in 100 cc. of phosphate buffer pH 7.4. The hydrochloride was neutralized with 2 cc. of 0.1 N sodium hydroxide. The cobaltous cysteine was then oxidized in each of two cells with the following oxidants respectively: air, ferricyanide 15 cc., 14.8 cc.; dibromophenolindophenol 12.8 cc., 12.8 cc.; hydrogen peroxide 14.0 cc., 15.0 cc. 2 × 10^{-4} mols of cysteine hydrochloride and 2 × 10^{-4} mols of sodium hydroxide were then added to each cell. The volume of 0.01 N indigo disulfonate required for each pair of duplicates, 2.0, 0.0, 0.5, and 2.0 cc. is a measure of the amount of cobalt ion which had not been converted to the complex by the first oxidant used. No cobalt was present after oxidation with ferricyanide as the ferrocyanide completely removed cobalt ion from solution.

Removal of Cobaltous Cysteine from Solution during Titration with Ferricyanide—Eight electrode cells were prepared all of which contained 2 × 10^{-4} mols of cysteine and 1 × 10^{-4} mols of cobalt sulfate. A 0.01 N solution of potassium ferricyanide which had been deoxygenated was added to the cells in the following amounts: 0, 1, 2, 3, 4, 5, 6, and 7 cc. respectively. The solutions were then titrated with 0.01 N indigo disulfonate. The volumes required

were 10, 8.5, 6, 4.5, 2.5, 1, 0.5, and 0.2. The cobaltous cysteine
had been removed from solution by the addition of ferricyanide
equal to two-thirds the concentration of cobaltous cysteine. The
amount of cobalt in these solutions was not sufficient to protect the
cobaltous cysteine from the action of the ferrocyanide. This ex-
periment was repeated with solutions which contained 2×10^{-4}
mols of cysteine and 2×10^{-4} mols of cobalt sulfate. The same
volumes of potassium ferricyanide were added. The volumes of
indigo disulfonate required to titrate the cobaltous cysteine were
10, 9, 8, 7, 6, 5, 4, and 1 cc. respectively.

*Oxidation of Cobaltous Cysteine by Addition of Cysteine and
Cobaltous Sulfate to Dibromophenolindophenol*—A solution which
contained 2×10^{-4} mols of cysteine hydrochloride and 1×10^{-4}
mols of cobalt sulfate was added drop by drop to 100 cc. of phos-
phate buffer pH 7.4 which contained 10, 20, and 30 cc. of 0.01 N
dibromophenolindophenol respectively. The solutions were de-
oxygenated. After the addition of each 5 cc. of the solution, which
contained cysteine and cobalt, 1 cc. of 0.10 N sodium hydroxide
was added to maintain the pH 7.4. The experiment was made in
duplicate. The following volumes of the solution of cobalt and
cysteine were used: 5.0, 5.0, 9.0, 9.5, 15.0, and 15.0. The equa-
tion for the oxidation of cobaltous cysteine to the complex and the
addition of cysteine to the dye is

$$x + 4 \times (1 - x) = \frac{\text{cc. of dye}}{\text{cc. of cobaltous cysteine}}$$

where x is the percentage of cobaltous cysteine converted to the
complex. x in each of the six solutions was: 66, 66, 59, 63, 66, and
66 respectively. By comparison of the intensity of color of the
first four solutions, after extraction of the dye with butyl alcohol,
with the color produced by oxidation of 10 cc. of 0.01 M cobaltous
cysteine with air, the percentage of cobaltic cysteine complex
formed by the dibromophenolindophenol was found to be 60, 63,
65, and 66 per cent, on the assumption that air converted 77 per
cent of the cobaltous cysteine to the cobaltic complex.

*Influence of Formaldehyde, Acetaldehyde, and Cabbage Juice on
Cobaltous Cysteine*—A solution of 2×10^{-4} mols of cysteine and
1×10^{-4} mols of cobalt sulfate in 100 cc. of phosphate buffer was
prepared. To this 5 cc. of 0.01 M formaldehyde were added. The

reduction potential rapidly became more positive and the color of the solution became much lighter. When the solution was exposed to air no cobaltic cysteine complex was formed. Acetaldehyde also brought about a decrease in reduction potential although the velocity of this reaction was much less than with formaldehyde. A solution of cabbage juice produced the same effect as formaldehyde.

Experiments with Glutathione—2×10^{-4} mols of glutathione which had been twice recrystallized from water were added to each of six cells all of which contained 100 cc. of phosphate buffer pH 7.4 and 2×10^{-4} mols of sodium hydroxide. To these solutions 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0×10^{-4} mols of cobaltous sulfate were added respectively. The addition of the cobalt salt did not increase the potentials in any of the solutions. The solutions remained water clear and colorless. 0.5 cc. of 0.01 N indigo disulfonate added to the solution was not reduced.

Experiments with Glutathione and Cystine in Presence of Cobalt— Five solutions were prepared which contained 100 cc. of buffer pH 7.4, 1×10^{-4} mols of cobalt sulfate, and 1×10^{-4} mols of cystine. To these solutions 0.2, 0.4, 0.8, 1.2, and 1.6×10^{-4} mols of glutathione were added. The brown color developed in solution was inversely proportional to the amounts of glutathione. The oxidation of the cobaltous cysteine was dependent on the ratio of cobaltous cysteine to cystine. 0.2, 0.8, 1.2, 1.6, and 4.1 cc. respectively of 0.01 N indigo disulfonate were reduced by the solutions. The cystine did not oxidize cobaltous cysteine to the complex in the higher concentrations of glutathione.

Oxidation of Cobaltous Cysteine with Oxidized Glutathione—Five cells were prepared which contained 100 cc. of phosphate buffer pH 7.4 and 0.0, 0.1, 0.2, 0.3, and 0.4 gm. of the neutral sodium salt of oxidized glutathione added in the form of a 1 per cent solution, 2×10^{-4} mols of cysteine hydrochloride, 2×10^{-4} mols of sodium hydroxide, and 1×10^{-4} mols of cobaltous sulfate freed from oxygen were added. The solutions were allowed to stand 24 hours and were titrated with indigo disulfonate. The volumes required were 6.0, 4.0, 3.5, 3.0, and 2.0 cc. respectively. The intensity of the color was proportional to the amount of oxidized glutathione added. The solution in Cell 5 was compared with 1×10^{-4} mols of cobaltous cysteine pH 7.4 oxidized with air. The

color was 125 per cent of the standard. This indicates almost complete oxidation of the cobaltous cysteine (see Table III). The color of the solution was not identical with the solution oxidized by air but an accurate comparison was possible.

Formation of a Cobaltic Complex with Cobalt Sulfate and Cysteyl Glycine (10, 12)—3 gm. of glutathione were dissolved in 100 cc. of water and the solution was maintained at 60° for 10 days. The solution was treated with mercury sulfate and the precipitate of the mercury salt of cysteyl glycine was decomposed with hydrogen sulfide. The volume was made to 50 cc. so that each cc. was equivalent to a 0.02 N solution of glutathione. 2, 4, 6, 8, 10, and 12 cc. of this solution were added to a series of six electrode cells which contained sufficient alkali to neutralize the cysteyl glycine. 1 × 10^{-4} mols of cobalt sulfate was added to each cell. The cobaltous thiol compound was oxidized to the cobaltic complex with the following volumes of 0.01 N indigo disulfonate: 0.5, 1.1, 2.9, 2.9, 3.8, and 5.6 cc. respectively. The solutions were light brown and resembled solutions of the cobaltic cysteine complex.

Oxidation of Cobaltous Cysteine Ester—The ethyl ester of cysteine was titrated with potassium ferricyanide in buffer at pH 7.4. 2 × 10^{-4} mols required the theoretical amount of this oxidant. The addition of 1 × 10^{-4} mols of cobalt sulfate to a solution of 100 cc. of phosphate buffer which contained 2 × 10^{-4} mols of the ethyl ester of cysteine produced an increase in reduction potential which was much higher than the corresponding potential of cobaltous cysteine. It reached the value −0.490 volt which is 44 millivolts negative to a hydrogen electrode at this pH.

The solution of cobaltous cysteine ester is colorless and after oxidation is a pale yellow since the oxidation product is only slightly soluble. Titration with ferricyanide oxidized the cobaltous cysteine ester to an insoluble dark colored product. The volume of ferricyanide required (14 cc. of 0.01 N solution) corresponded closely to the volume required for cobaltous cysteine. The volume of indigo disulfonate required was 10 cc. which is the amount reduced by 1 × 10^{-4} mols of cobaltous cysteine.

Reaction of Cobaltous Cysteine with Sodium Sulfide, Hydrosulfite, Sulfite, and Thiosulfate—A solution which contained 2 × 10^{-4} mols of cysteine and 1 × 10^{-4} mols of cobalt sulfate, was prepared in each of four electrode cells. 9 cc. of 0.009 N sodium sulfide, 22 cc.

of 0.005 N sodium hydrosulfite in 0.01 N sodium hydroxide, 10 cc. of 0.01 N sodium sulfite, and 10 cc. of 0.01 N sodium thiosulfate were added to the cells respectively, in several small portions.

Sodium sulfide produced a black precipitate of cobalt sulfide. The reduction potential was not decreased. Sodium hydrosulfite at first produced a marked increase in the reduction potential which gradually returned to a value which was more positive than before the addition of the hydrosulfite. The color of the solution was the same as that of a solution of the cobaltic cysteine complex. Sodium sulfite after 18 hours brought about oxidation of the cobaltous cysteine but the potential still indicated the presence of some cobaltous cysteine and the solution reduced 1 cc. of 0.01 N indigo disulfonate. This is 10 per cent of the amount required for complete oxidation of the cobaltous cysteine. Sodium thiosulfate did not modify the color or potential of the solution of cobaltous cysteine. Sodium sulfide, added to a solution of cobaltic cysteine complex, did not modify the color and did not precipitate cobalt sulfide from solution. If hydrogen sulfide is passed through a solution of the brown cobaltic complex made slightly acid with hydrochloric acid, the solution loses its intensity of brown which indicates the decomposition of the cobaltic complex. Acetic and hydrochloric acids in small amount do not modify the color of the cobaltic cysteine complex.

Determination of Intensity of Color and Amount of Cystine Formed—The amount of the brown cobalt cysteine complex was determined in a colorimeter. There was no change in the intensity of color even after standing for 48 hours. The intensity of color when oxygen was used could be duplicated within 5 per cent. When dibromophenolindophenol was the oxidant it was necessary to remove the dye. The solution was extracted four times with butyl alcohol which was saturated with water. The butyl alcohol was removed by two extractions with ether and after rapid aeration the original volume of the solution was restored. The cobaltic complex is insoluble in butyl alcohol and ether. Both the oxidized and leuco form of dibromophenolindophenol are soluble in butyl alcohol.

For the determination of cystine the following modification of Folin and Looney's (5) method was used. The standard was a solution of 0.5 N hydrochloric acid which contained 1 mg. of cystine

for each cc. 1 cc. was placed in a 100 cc. volumetric flask and 38.5 cc. of water were added.   7.5 cc. of N sodium hydroxide (0.5 cc. to neutralize the acid in the standard) and 10 cc. of 15 per cent sodium sulfite were added to the flask, 3 cc. of Folin and Trimble's (6) uric acid reagent were added and the color was allowed to develop for 10 minutes.   The solution was then diluted to the 100 cc. mark and compared with the unknown which was prepared in the same manner, in respect to volumes of reagents and the volume in which the color was developed.

The presence of cobalt interferes with the reduction of sodium phosphotungstate.   It was therefore necessary to remove completely all traces of cobalt.   This was done as follows: 20 cc. of the solution from the electrode cell which contained the cobaltic cysteine complex, cystine, and an excess of cobaltous salt were placed in a flask and to this 1 cc. of 0.01 M cobalt sulfate, 3 cc. of N sodium hydroxide, and 1 cc. of 0.10 N hydrogen peroxide were added. The volume was made to 30 cc. and after an interval of 15 minutes the cobaltic hydroxide was filtered from the solution through a dry filter into a dry flask.   15 cc. of the filtrate, which was equal to 10 cc. of the original solution, were then diluted to 40 cc.   Since this solution contained 1.5 cc. of N sodium hydroxide, 5.5 cc. of N sodium hydroxide were added, to make a total of 7 cc. of N sodium hydroxide.   10 cc. of 15 per cent sodium sulfite and 3 cc. of the uric acid reagent were then added.   The standard was prepared by the addition of 2 cc. of the standard solution to 20 cc. of buffer which contained 1 cc. of 0.01 M cobalt sulfate.   The solution was then treated as 20 cc. of the solution from the electrode cell.

For the removal of indigo disulfonate 150 mg. of activated charcoal (carboraffin) and 1 cc. of 5 N sulfuric acid were added for each 100 cc. of solution which contained the cobaltic cysteine complex. The solution was filtered and cystine determined in 10 or 20 cc. of the filtrate after the cobalt had been removed.   It was shown that when 150 mg. of activated charcoal were added to 100 cc. of a solution of phosphate buffer which contained the same amount of acid and 1 mg. of cystine, 93 per cent of the cystine was in the filtrate.

For the removal of ferrocyanide 12 cc. of 0.01 M cobaltous sulfate and 1 cc. of 50 per cent acetic acid were added to 50 cc. of the solution from the electrode cell.   Cobaltous ferrocyanide sepa-

rated and was filtered from solution. The filtrate was made neutral with 3 cc. of N sodium hydroxide and cystine was determined in 20 cc. of the solution after the cobalt has been removed as already described.

For the removal of dibromophenolindophenol 100 cc. of the solution were extracted four times with 20 cc. of butyl alcohol. Crystalline quinone in excess was then added to the solution. This resulted in the oxidation of some of the addition product between cysteine and the dye which was not extracted with butyl alcohol. After 5 minutes the solution was dark red. 1 cc. of 5 N sulfuric acid was added and the solution extracted ten times with butyl alcohol. Quinol and almost all of the reducing substances other than cystine were extracted from the solution. Cystine is not affected by the treatment with quinone nor with butyl alcohol. The butyl alcohol was removed with ether. The ether was removed by aeration and the original volume restored. 100 cc. of this solution were treated with 150 mg. of carboraffin. The solution was filtered and cystine determined in 10 or 20 cc. portions of the filtrate. In case cobalt was present it was removed as described.

When the color is developed in a volume of 40 cc. the uric acid reagent gives a slight blue color in the absence of cystine which is equivalent to about 0.25 mg. of cystine. For the determination of cystine after oxidation of cobaltous cysteine with indigo disulfonate a more accurate method was desired. For these solutions the color was developed in a volume of 100 cc. and the entire solution of the electrode cell was used, after the dye, unchanged cysteine, and any trace of cobalt had been removed. Before any oxidation of the excess cysteine could occur solid quinone (75 to 100 mg.) was added and the solution stirred.

After 5 minutes, 2 cc. of 5 N sulfuric acid were added and the solution was extracted four times with butyl alcohol, three times with ether, and aerated. 150 mg. of carboraffin were added and the solution filtered through dry paper into a dry flask. 3 cc. of 5 N sodium hydroxide, 2 cc. of 0.01 M cobalt sulfate, and 1 cc. 0.1 N hydrogen peroxide were added. After 15 minutes the solution was filtered through dry paper into a dry flask. 5 cc. of N sodium hydroxide, 10 cc. of 15 per cent sodium sulfite, and 3 cc. of uric acid reagent were added. After 10 minutes the solution was compared

with a standard prepared by the addition of 1 mg. of cystine and 10 cc. of 0.01 N indigo disulfonate to 100 cc. of phosphate buffer. The solution was treated as described. When the color is developed in this volume a blank is secured in the absence of cystine. A precipitate of potassium phosphotungstate forms but this can be removed by the centrifuge.

Determination of Cystine in Solutions of Cobaltous Cysteine Oxidized with Indigo Disulfonate—The solutions which were obtained from the experiment charted in Fig. 11 were treated with quinone, activated charcoal, and butyl alcohol as described. Only a faint trace of color was given in the solutions which did not contain cysteine in excess of twice the molar equivalent of cobaltous sulfate. In solutions which contained an excess of cysteine a positive test for cystine was given. This was in marked contrast to the solutions that contained cobalt in excess. The accurate determination of cystine was not possible but in no case was there more than 1 mg. present in the entire solution.

Evidence of a Time Reaction in the Oxidation of Cobaltous Cysteine —Four cells were prepared all of which contained 2×10^{-4} mols of cysteine hydrochloride, 2×10^{-4} mols of cobalt sulfate, 2×10^{-4} mols of sodium hydroxide, and 100 cc. of buffer pH 7.4. Cell 1 was titrated rapidly with 0.01 N potassium ferricyanide. Cell 2 was titrated slowly with the same oxidant. 5 cc. of 0.01 M cobalt sulfate were added to Cell 3, then 6 cc. of 0.01 N ferricyanide were added drop by drop. 5 cc. more of the cobalt solution were added and the titration was completed slowly with ferricyanide. 10 cc. of 0.01 M cobalt sulfate were added to Cell 4 and the solution was then slowly titrated with 0.01 N ferricyanide. The volumes·required were 13.5, 14.5, 12.0, and 12.8 cc. respectively.

Factors Which Influence Available Cobalt Ion—Glutathione added to cobaltous cysteine interferes with the formation of the cobaltic complex. Five electrode cells were prepared all of which contained 1×10^{-4} mols of cobaltous cysteine in 100 cc. of buffer pH 7.4. 0.0, 0.5, 1.0, 1.5, and 4.5×10^{-4} mols of glutathione were added respectively. After 4 hours the cobaltous cysteine was titrated, the following volumes of 0.01 N indigo disulfonate were required: 10, 9, 9.5, 9.5, and 8.0 cc. respectively.

TABLE III

Amount of Cobaltous Cysteine Oxidized to Cobaltic Cysteine Complex and to Cystine in Relation to the Volume of Oxidant Required

Oxidant	0.01 N cysteine	0.01 M cobalt sulfate	Volume of 0.01 N oxidant	Color in per cent of Solution 1	Cystine formed	Remarks
	cc.	cc.	cc.		mg.	
Air						
Solution 1	20	20		100	6.6	
" 2	20	10		100	5.5	
" 3	20	10		100	5.6	
" 4	20	10		100	6.1	
" 5	20	11		100	5.6	
" 6	20	10		100	5.5	
" 7	20	11		51	10.4	} Cobaltous cysteine added to aerated buffer
" 8	20	10		53	10.7	
" 9	20	10		64	11.7	
" 10	20	10		57	9.3	
Hydrogen peroxide						
Solution 11	20	20	13	102	6.25	} Added slowly
" 12	20	20	13	102	6.25	
" 13	20	10	13.3	106	5	
" 14	20	11	13	93	6	} Added rapidly
" 15	20	11	13.2	96	6.2	
" 16	20	11	13.5	94	6.1	
" 17	20	10	13	101	6	4 cc. added rapidly, titration finished slowly
" 18	20	10	14	91	6.3	8 cc. added rapidly, titration finished slowly
" 19	20	10	20	92	6.6	12 cc. added rapidly, titration finished slowly
" 20	20	10	13	102	6	Added slowly
" 21	20	11	20	44	8.8	} Solution of cobalt and cysteine added to hydrogen peroxide
" 22	20	11·	20	45	9.1	
" 23	20	10	20	65	8	
" 24	20	10	20	80	6.6	
Ferricyanide						
Solution 25	20	20	13.3		8.5	
" 26	20	10	15		11.7	

TABLE III—*Concluded*

Oxidant	0.01 N cysteine	0.01 M cobalt sulfate	Volume of 0.01 N oxidant	Color in per cent of Solution 1	Cystine formed	Remarks
	cc.	*cc.*	*cc.*		*mg.*	
Dibromophenol-indophenol						
Solution 27.....	20	0	40		Trace	
" 28.....	20	0	40		"	
" 29.....	20	11	13.3	111	"	
" 30.....	20	11	13.3	118	"	
" 31.....	20	20	13	110	"	
Indigo disulfonate						
Solution 32.....	20	10	10		Trace	
" 33.....	20	10	10		"	
Cystine						
Solution 34.....	20	10	160	133		After 24 hrs. did not reduce indigo disulfonate
Sodium sulfite						
Solution 35.....	20	10	100	111		After 24 hrs. reduced 1 cc. 0.01 N indigo disulfonate

TABLE IV

Comparison of Amounts of Cobaltous Cysteine Converted to Cobaltic Complex and to Cystine as Determined by Titration and by Intensity of Color

Oxidant	Cobalt complex*	Cobalt complex†	Cystine
Ferricyanide.....................	66		33
Oxygen.........................		77	23
Hydrogen peroxide...............	77	77	23
Dibromophenolindophenol........	89	87	Trace
Sodium sulfite..................	90	85	
Cystine........................	100	101	
Indigo disulfonate..............	100	100‡	Trace

* Per cent of cobaltous cysteine converted to complex determined by titration.

† Per cent of cobaltous cysteine converted to complex determined by color comparison. Intensity of color developed by hydrogen peroxide taken as standard.

‡ Estimated.

SUMMARY

Oxidation of cobaltous cysteine has been quantitatively studied with the following oxidants: indigo disulfonate, ferricyanide, dibromophenolindophenol, hydrogen peroxide, and oxygen. 2 molecules of cysteine combine with 1 atom of cobalt and require 1 equivalent of an oxidant for the formation of a brown cobaltic cysteine complex.

Indigo disulfonate is the only oxidizing agent which brings about a quantitative conversion of cobaltous cysteine to the cobaltic complex. The amounts of ferricyanide, dibromophenolindophenol, hydrogen peroxide, and oxygen required to oxidize cobaltous cysteine as shown by Michaelis and coworkers, are for each oxidant two-thirds the concentration of the thiol group. This is coincidence since none of the oxidants convert cobaltous cysteine quantitatively into the cobaltic complex and there is a different side reaction with each oxidant. With ferricyanide the formation of ferrocyanide decomposes cobaltous cysteine into cobalt ferrocyanide and cysteine. The cysteine is oxidized to cystine. Even with excess of cobalt one-third of the thiol group is oxidized to cystine and two-thirds to the cobaltic cysteine complex.

Hydrogen peroxide and oxygen convert 77 per cent of the cobaltous cysteine into the cobaltic complex and 23 per cent to cystine. Dibromophenolindophenol converts 89 per cent into the cobaltic complex, 11 per cent of the thiol group combines with the quinone group of the dye. Cysteine and cobalt sulfate added to dibromophenolindophenol or to ferricyanide results in the oxidation of two-thirds of the thiol group to the cobaltic cysteine complex.

Cystine, oxidized glutathione, sodium hydrosulfite, and sodium sulfite convert cobaltous cysteine into a cobaltic cysteine complex. Sodium thiosulfate does not convert cobaltous cysteine into a cobaltic complex.

Formaldehyde, acetaldehyde, and cyanide decompose cobaltous cysteine and prevent its oxidation to the cobaltic complex.

Determination of the intensity of the color of the brown cobaltic complex confirms the results obtained by titration of the cobaltous cysteine with indigo disulfonate.

The reduction potential of cobaltous cysteine increases slowly for many hours. The addition of a small amount of oxidant cata-

lyses this reaction and the potential is immediately increased to its maximal.

The hypothesis is made that an intermediate cobaltic tricysteine salt is first formed. This rearranges into the cobaltic dicysteine complex and a negatively charged $-SR$ group. The quantitative relationships between cobaltous cysteine and the various oxidants may be explained by differences in the velocities of four reactions which are involved in the oxidation of cobaltous cysteine.

Indigo disulfonate cannot oxidize cysteine to cystine. A small percentage of the total cysteine is converted into cystine with indigo disulfonate if cobalt is present in less than half the molar concentration of the cysteine.

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THE EFFECT OF FASTING ON THE CREATINE AND NITROGEN CONTENT OF THE BODY AND MUSCLE OF THE WHITE RAT

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A review of the literature (1) shows a paucity of data concerning the concentration of chemical constituents of the whole organism during fasting. The experimental work is limited for the most part to changes noted in individual tissues. Furthermore, it seems difficult to draw any definite conclusion concerning the changes in the composition of the organism because of the varying results obtained in so many different types of experimental animals.

Recent work has shown an increased percentage creatine concentration in several tissues of the fasting white rat (2). It may be assumed that the percentage concentration of creatine would increase in the whole organism, particularly since the muscle shows the greatest increase. In view of these considerations it was deemed important to study further the creatine concentration of the normal and fasting whole white rat (eviscerated). In addition observations of concomitant changes in body constituents other than creatine were made.

Methods

Mature albino rats were used for experimentation. Since early analyses showed no perceptible percentage differences in the constituents studied between the two sexes, the sex is omitted in the experimental data. Before the fast was begun, the animals were kept on a stock diet.[1] During the period of fasting, the rats

[1] Bal Ra, a dog food preparation prepared by the Valentine Meat Juice Company of Richmond, Virginia, was used.

were placed in individual double bottomed mesh cages to avoid coprophagy. Water was always available. The methods for analysis of the constituents studied in the eviscerated animals have been described in a previous paper (3).

Experimental Results

The data summarized in Table I reveal the following points. (1) The percentage concentration of ether extract and total solids decreases progressively during fasting. (2) The percentage of ash increases appreciably. (3) Creatine calculated on the basis of dry, fat- and ash-free tissue shows a remarkable constancy during all stages of fasting. (4) The creatine content of fresh tissue increases. (5) Nitrogen calculated in the same manner as creatine is constant until the animal is about ready to die, at which time there is an increase.

It is seen that the drop in the percentage of total solids is due primarily to the loss of fat. When the organic material is calculated by subtracting the ash and fat from the total solids, it is found that the difference is practically negligible during the various stages of fasting, the percentage changing from 21.2 for the control to 24.0 for the animals exposed to the longest fast.

In studying the changes of the constituents of the body during fasting or in any condition in which there is a variation or change in body weight, it becomes extremely important to take into consideration not only total solids but also ash and fat. The results obtained in this work through a consideration of these factors have yielded surprising results, particularly in regard to the concentration of creatine and nitrogen in the body. A relationship between these two constituents is suggested in this work. In all probability this fact is more accidental than true for the creatine content of many of the organs in which the greatest loss of weight is noted (4) is comparatively small. This same reasoning would apply to any suggested relationship between urinary creatine and creatinine and total nitrogen. We feel that there is no evidence to suggest any metabolic parallelism between creatine and nitrogen metabolism. This same conclusion was reached by Benedict and Osterberg (5) in their work with phlorhizinized dogs.

The data dealing with the effect of fasting upon various constituents of muscle are summarized in Table II. This work was done

primarily to check the creatine data previously published (2) and in addition to study the nitrogen of muscle. The creatine

TABLE I

*Effects of Fasting on Various Components of the Eviscerated White Rat**

	No. of rats	Initial weight	Final weight	Eviscerated weight	Weight lost	Length of fasting	Ether extract	Ash	Total solids	Total solids − fat and ash (organic)	Total N	Total N in dry, fat- and ash-free tissue	Total creatine	Total creatine in dry, fat- and ash-free tissue
							Gm. per 100 gm. tissue (per cent)						

| | | | | | | | Control group | | | | | | |

		gm.	gm.	gm.	per cent	days								
Minimum...	172		156				4.4	3.18	32.8		3.18		0.200	
Maximum..	332		305				22.3	4.52	45.7		3.68		0.282	
Average....	39	230		224			13.4	3.81	38.4	21.2	3.37	15.9	0.234	1.10

| | | | | | | | 20 to 30 per cent body weight lost | | | | | | |

Minimum...		161	120	111	6		2.23	3.94	30.6		3.33		0.203	
Maximum..		325	260	247	15		14.30	5.15	40.3		3.91		0.267	
Average....	25	230	171	161	25.8	9.8	8.47	4.53	35.2	22.2	3.53	15.9	0.240	1.08

| | | | | | | | 30 to 40 per cent body weight lost | | | | | | |

Minimum...		163	109	104	9		0.80	4.10	28.7		2.93		0.225	
Maximum..		415	290	280	19		10.40	6.68	37.4		4.34		0.288	
Average....	49	239	158	142	33.9	13.6	5.28	4.99	33.5	23.2	3.68	15.9	0.254	1.09

| | | | | | | | 40 to 50 per cent body weight lost | | | | | | |

Minimum...		181	102	96	14		0.74	4.57	30.5		3.86		0.242	
Maximum..		274	157	149	15		5.50	7.04	33.9		4.67		0.294	
Average....	15	239	134	129	42.2	14.7	2.44	5.64	32.1	24.0	4.05	16.8	0.264	1.10

* The gastrointestinal tract was removed.

content of the muscle of the control animal is slightly higher than previous figures obtained in this laboratory. However, there is an unquestionable increase in the creatine concentration in the

muscle of the fasting animal, which confirms previous observations. It should be noted that this increase holds true for the fresh and "organic" muscle. On the other hand, the percentage concentration of nitrogen in the muscle does not change during fasting. It is difficult to correlate an increase in percentage concentration of muscle creatine with the apparent constancy of body creatine.

The total solids decrease in the muscle as they do in the whole body. The ash determinations were omitted after it was found by preliminary determinations that this constituent changed very little with fasting.

TABLE II

Effect of Fasting upon Several Constituents of Muscle

		No. of rats	Total solids	Fat	Total creatine	Creatine in dry and fat-free tissue	Total N	N in dry and fat-free tissue
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Control	Minimum		24.2	1.23	0.457	2.04	3.37	14.4
	Maximum		26.2	3.30	0.507	2.28	3.49	15.8
	Average	12	24.8	2.11	0.488	2.15	3.43	15.1
30 to 40 per cent body weight lost	Minimum		23.0	0.84	0.493	2.22	3.38	15.1
	Maximum		24.1	2.40	0.529	2.38	3.57	15.9
	Average	11	23.7	1.34	0.513	2.30	3.46	15.2

A detailed analysis of the changes in the weight of the individual constituents during various stages of fasting are presented in Chart I. The average initial weights of the animals in the various groups are approximately the same, thus making a comparison fairly accurate. The weights of the constituents were obtained by multiplying the weight of the animal by its percentage concentration.

As a result of extreme inanition, the organism loses 89.5 per cent of its body fat. The loss of fat seems to be fairly rapid and to a certain extent in proportion to the loss of body weight. The total solids show a decrease of 52.2 per cent. This marked decrease is due in large measure to the marked diminution in the fat content. In fresh tissue there is a loss of 31.2 per cent of the

total nitrogen and 35.4 per cent of the creatine.   In the "organic" carcass there is a decrease of 40.0 per cent of total nitrogen and 42.8 per cent of the creatine.   The organic content of the organism
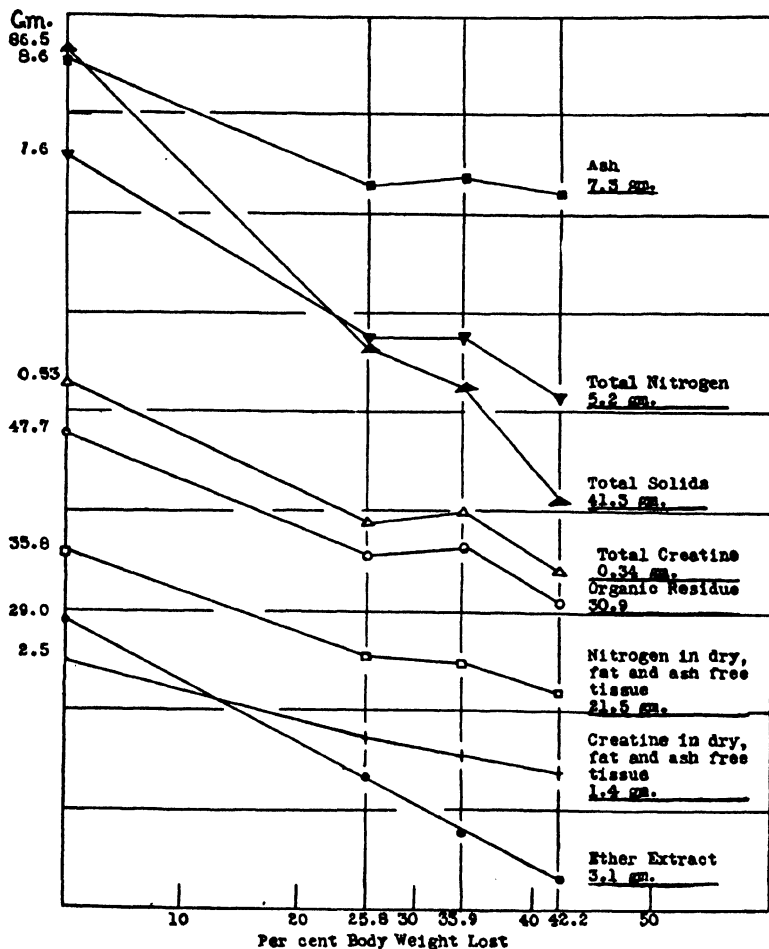
CHART I. Graphic representation of the changes in the weight of different components of the whole white rat during fasting.

is decreased 35.3 per cent.   The loss of ash is relatively small (15.1 per cent) as is to be expected.

It would be extremely interesting to obtain accurate results concerning the total nitrogen, creatine-creatinine, and the salt

output in the urine of the fasting rat. Preliminary attempts at such a study presented many difficulties because of the extremely small amounts of urine excreted by the fasted animal.

It was interesting to compare the averaged data obtained by Myers and Fine (6) in rabbits with those of the rat. They noted a loss of 45 per cent of body creatine in seventeen rabbits that had lost 45 per cent of their body weight. On the basis of the analyses of fresh muscle, the rabbit loses approximately 10 per cent more creatine than the rat under similar conditions of acute inanition.

In attempting to explain any phase of creatine metabolism, the rather striking constancy of the percentage concentration of creatine in the organism during fasting and during the administration of a wide variety of diets must be taken into consideration. Furthermore, the immediate creatine saturation of the organism after the feeding of a high creatine diet is significant (3). There apparently is a definite physiological mechanism for maintaining a constant level of creatine storage in the body. The urinary and blood findings in various experimental conditions serve to show wide metabolic variations in attaining this end. This is illustrated by the creatinurias of high protein feeding, poor nutritive conditions, fasting, and the growth period; the wide variations in the urinary picture obtained after feeding various amounts of creatine; the increased blood concentration of creatine and creatinine in nephritis. In addition the unknown factors pertaining to the endogenous metabolism must be considered.

SUMMARY

The percentage creatine concentration of the whole "organic" white rat (eviscerated) remains constant during the entire period of fasting. The nitrogen concentration increases slightly when the animal has lost 40 to 50 per cent of its body weight. Data are presented to show the changes in the total solids, fat, and ash concentration during various stages of fasting.

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STUDIES IN GASTRIC SECRETION

II. A COMPARISON OF CRITERIA OF ACIDITY USED IN THIS INVESTIGATION

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When the present series of investigations was first undertaken (Hollander and Cowgill, 1931), it was thought that an electrometric determination of pH values would be more important as a criterion of acidity in pure gastric juice than the usual titrimetric determination of free and total acidities. This was so because of the possibility that the intracellular mechanism by which gastric hydrochloric acid is formed may depend on a membrane hydrolysis of neutral chlorides, in accordance with the Donnan theory of membrane equilibria, or, more likely, according to concepts of specific ionic permeabilities. In either case, the hydrogen ion activity would undoubtedly be the significant factor, especially if considerable amounts of neutral salts and acid-binding proteins are present, as is suggested by the observations of other investigators. Accordingly, in the beginning, the micro quinhydrone technique described below was employed.

As the work progressed, however, some doubt arose concerning the desirability of pH values in this connection. The greatest possible reliability of their determination corresponds to 0.01 pH units. Now, for concentrations of acid in the neighborhood of 150 mM (pH 0.9), a difference of this magnitude corresponds to 3 mM; *i.e.*, although the experimental variation in terms of pH is gratify-

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ingly small, in terms of molarity it is about 2 per cent of the entire concentration.   On the other hand, duplicate determinations by micro titration will rarely show an average deviation of more than 0.3 per cent.   Consequently, under the conditions which obtain in this work, the titrimetric method is more sensitive than the electrometric—contrary to the usual experience with body fluids. Further, following observations already reported in Paper I of this series, it seemed unlikely that under these conditions there occur any important variations in the activity coefficient of the gastric juice, resulting from large changes in its content of salt and protein.   Thus, a major reason for the use of pH as a criterion of acidity in the first place was removed.   Finally, it became apparent that changes in the concentrations of HCl and its salts in the original parietal secretion, resulting from neutralization and dilution by mucus, peptic secretion, etc., were worthy of much attention.   Obviously, a study of ˙these phenomena necessitated a consideration of anion and cation concentrations in terms of molarity.   For these reasons, therefore, it was concluded that the electrometric criterion of acidity previously employed ought to be replaced by a carefully controlled titrimetric procedure, and the micro technique described below was used in all subsequent work.

This conclusion, however, presented a question regarding the equivalence of pH and titration acidities in gastric secretion studies.   Fairly good agreement of the two (on stomach contents) was reported by Tangl (1906) with Congo red as indicator, and by Shohl and King (1920) with both thymol blue and Töpfer's reagent.   Similar concordance in pouch juice was reported by Fraenckel (1905) when Congo red was employed.   On the other hand, Foà (1905), who also used Congo red, and Rosemann (1917), who used phenolphthalein, both reported significant divergences with pouch juice.   Likewise, Michaelis and Davidsohn (1910–11) obtained disagreeing values on stomach contents with methyl orange as the indicator.   A careful investigation of the entire problem by Christiansen (1912) indicated that none of the indicators commonly used for determining acidity by titration in stomach contents (except Günzberg's reagent) gave values which were concordant with the results of pH determinations.

In view of the uncertainty prevailing on this point, simultaneous determinations of pH and of free and total acidities were performed

on a series of gastric juice samples. These specimens were obtained from several stomach pouch dogs by either the continuous or the discontinuous collection procedures previously described (Hollander and Cowgill, 1931). Either histamine or one of a variety of foods was used as a stimulus. The observed values are given in Columns 2, 3, and 6 of Table I.

In order to compare the two methods, it was necessary first to convert the titrimetric acidities into the corresponding pH values (Columns 4 and 7). This change was effected by means of the formula.[1]

$$pH = \log \frac{\gamma_{KCl}}{\gamma^2_{HCl} \times m}$$

where γ_{KCl} and γ_{HCl} are the activity coefficients for KCl and HCl at a molar concentration of m. Values for these coefficients at 25° are given by Lewis and Randall (1923) for m equal to 0.05, 0.1, and 0.2. Intermediate values were determined from these by interpolation. Finally, the differences between the observed values of pH and those calculated in this way are given in Columns 5 and 8. For total acidity, these values are all positive or zero; the average of the thirty numbers is 0.013. The free acidity differences, however, show a random distribution about zero, with an average value of 0.006 pH.

Several reasons for this excellent agreement between pH and free acidity, in contrast with the observations of others, suggest themselves. (1) The samples of gastric juice studied were all collected under the carefully controlled conditions previously established. Such secretion is of a very high degree of purity; neutral chlorides and acid bound by proteins are both extremely low (Hollander, 1928). Therefore, the activity coefficient in such a gastric hydrochloric acid solution is practically identical with that for the pure acid. For the same reason, the progressive dissociation of protein hydrochloride which occurs in the free acidity titration of stomach contents (Christiansen, 1911) is practically absent

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[1] $pH = \log \dfrac{1}{\alpha_H} = \log \dfrac{1}{\gamma_H \times m}$. But $\gamma_H = \dfrac{\gamma^2_{HCl}}{\gamma_{Cl}}$ and $\gamma_{Cl} = \gamma_K = \gamma_{KCl}$.

Therefore, $pH = \log \dfrac{\gamma_{KCl}}{\gamma^2_{HCl} \times m}$.

from this fluid.   (2) In calculating pH values from the observed
E.M.F. readings, or molar concentrations from these pH values,

TABLE I

Comparison of Electrometric and Titrimetric Acidity Values

Sample No.	pH (determined)	Free acidity			Total acidity		
		mM (determined)	pH (calculated)	(2) − (4)	mM (determined)	pH (calculated)	(2) − (7)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	0.91	0.148	0.91	0	0.152	0.90	+0.01
2	0.89	0.152	0.90	−0.01	0.156	0.89	0
3	0.91	0.152	0.90	+0.01	0.158	0.88	+0.02
4	0.92	0.141	0.93	−0.01	0.147	0.92	0
5	0.92	0.146	0.92	0	0.150	0.91	+0.01
6	0.97	0.130	0.97	0	0.141	0.93	+0.04
7	0.93	0.141	0.93	0	0.150	0.91	+0.02
8	0.96	0.136	0.95	+0.01	0.146	0.92	+0.04
9	0.93	0.136	0.95	−0.02	0.141	0.93	0
10	1.01	0.123	0.99	+0.02	0.132	0.96	+0.05
11	0.90	0.156	0.89	+0.01	0.160	0.88	+0.02
12	0.89	0.159	0.88	+0.01	0.160	0.88	+0.01
13	0.90	0.156	0.89	+0.01	0.158	0.88	+0.02
14	0.89	0.158	0.88	+0.01	0.160	0.88	+0.01
15	0.91	0.148	0.91	0	0.151	0.90	+0.01
16	0.95	0.131	0.96	−0.01	0.135	0.95	0
17	0.92	0.145	0.92	0	0.147	0.92	0
18	0.94	0.137	0.95	−0.01	0.141	0.93	+0.01
19	0.99	0.122	0.99	0	0.126	0.98	+0.01
20	0.95	0.134	0.95	0	0.137	0.95	0
21	0.94	0.138	0.94	0	0.140	0.94	0
22	1.14	0.089	1.12	+0.02	0.096	1.10	+0.04
23	0.98	0.127	0.98	0	0.131	0.96	+0.02
24	0.97	0.128	0.98	−0.01	0.132	0.96	+0.01
25	0.95	0.135	0.95	0	0.138	0.94	+0.01
26	0.93	0.142	0.93	0	0.145	0.92	+0.01
27	0.96	0.131	0.96	0	0.137	0.95	+0.01
28	0.95	0.134	0.95	0	0.137	0.95	0
29	0.89	0.159	0.88	+0.01	0.160	0.88	+0.01
30	0.91	0.150	0.91	0	0.153	0.90	+0.01

many investigators have used an older, less accurate pH value for
0.1 M HCl as a standard of reference instead of 1.08 as given by
Lewis and Randall.   For instance, Christiansen (1912) adopted

pH 1.00, whereas Michaelis and Davidsohn (1910-11) used pH 1.04 which they derived on the basis of 91 per cent as the fraction ionized. The error introduced in this way might amount to 10 or more mm. (3) Finally, most of the previous workers have performed their titrations in the usual way, without any attempt to control exactly the final pH of the end-point as has been done here. This also would introduce a discrepancy, particularly in the free acidity value where even small amounts of buffer will exert a marked influence on the volume of alkali required.

It is to be concluded, therefore, that titration acidities, determined under the conditions here established, will give the same information afforded by electrometric pH determinations, and more besides.

Methods

pH Determination

Since it frequently happened that only small quantities of gastric juice were available for duplicate pH determinations, it was necessary to employ a micro method. Further, because of the large number of such measurements required for each entire experiment, it was essential that the method be rapid and easy of performance. A preliminary study having shown that the principle of the quinhydrone electrode was satisfactory in all respects, it was employed in preference to the gas or other electrode. At the time this work was started, none of the methods found in the literature was suitable to these ends, as a result of which the following very simple system, requiring not more than 0.2 cc. for each determination, was devised.

Apparatus—The electrode vessel is a small tapered glass tube (Fig. 1). The narrowest part, the stem, *A*, is filled with a KCl-agar plug which serves as a junction between the KCl bridge and the liquid to be measured when the cell is placed into the former. In this way, even the slightest mixing of unknown liquid and KCl is prevented. The body, *B*, is made conveniently small, so as to minimize the quantity of fluid necessary to establish electrical contact between the platinum electrode and the agar surface. In order to give firm support to the electrode, the vessel is expanded into the head, *C*, which is provided with a slight flange at the mouth. The electrode, *D*, itself is constructed of about 13 cm. of

narrow glass tubing (3 to 4 mm. outer diameter) with 6 to 8 mm. of number 26 gage platinum wire[2] sealed into one end. To facilitate mixing the quinhydrone with the liquid under examination, the

DIAMETER 7 MM. → C

D

25 MM.

DIAMETER 5 MM. → B

12 MM.

DIAMETER 2.5 MM. → A

20 MM.
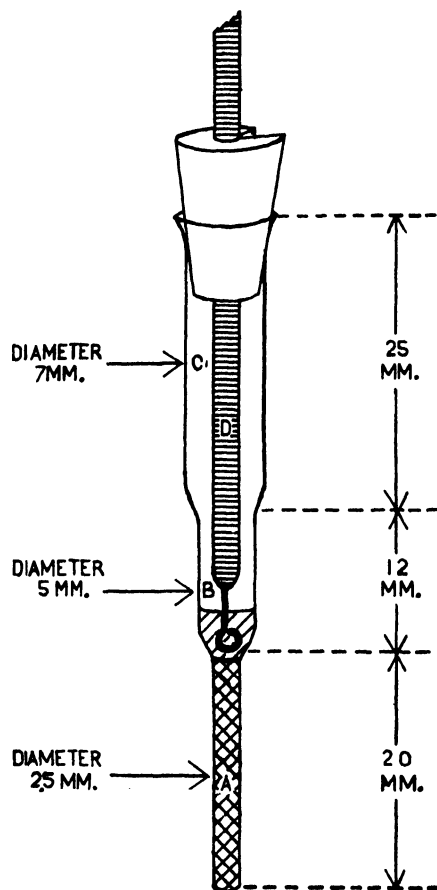
FIG. 1. Micro quinhydrone electrode vessel. *A* is the stem filled with a KCl-agar plug; *B*, the body, contains the fluid to be measured; and *C*, the head, supports *D*, the electrode.

end of the platinum wire may be twisted into a single narrow loop as indicated. Electrical contact with a copper wire is established

[2] Gold wire gave very unsatisfactory results. The efficiency of gold-placed platinum was not investigated.

through mercury in the usual way. The electrode tube can be held firmly in the mouth of the vessel by means of a small paraffined cork. A narrow wedge cut out of the cork makes it possible to insert it without forcing the agar plug through the stem.

In order to make eight or ten determinations simultaneously, the saturated KCl bridge must be large enough to accommodate at least this many cells besides the saturated KCl-calomel cell. A convenient arrangement consists of a cylindrical jar, 10 cm. in diameter and 8 to 10 cm. deep, which is provided with a papier-maché cap to support the cells. This cover should contain about a dozen holes, large enough to permit passage of the electrode vessel up to, but not including, its flange. A diameter of 10 mm. will be found adequate for the purpose. To facilitate the manipulation requisite for the simultaneous determination of a number of pH values, the leads from the individual quinhydrone cells can be connected to the potentiometer through a distributing switch. E.M.F. measurements are made as usual.

Manipulation—After thoroughly cleaning and drying the electrode vessel, its stem is filled with a KCl-agar plug. The gel can be easily made by the slow addition, with gentle heating, of 3 per cent of pulverized agar to a hot solution which has been saturated with KCl at 25°. This will yield a firm gel at room temperature, the solidification of which will not occur too rapidly to complicate its manipulation. In fact, it has been found that when this mixture is kept at 60–70° it remains sufficiently fluid to enable the stem to be filled to just below the shoulder simply by immersion to this level. On the other hand, solidification will occur quite readily merely by contact with the cool glass, since holding the vessel in the agar for 30 seconds is usually sufficient to prevent any slipping of the gel when the stem is removed and held vertically. Whenever the initial temperature of the agar is much above 70°, such slipping will invariably occur, leaving a coat of KCl-agar on the wall of the vessel above the surface of the plug. Rapid crystallization of KCl at this surface will frequently cause a failure of reproducibility in the E.M.F. reading. To prevent excessive loss of water on frequent reheating, the stock solution of agar should be divided into 50 cc. lots, each lot to be used not more than three or four times. Ordinarily, only about a dozen plugs can be made up at one time, for whenever these are kept for more than a few hours

before using, crystallization results with consequent irregularity in potentiometer readings.

By means of a piece of glass tubing drawn to a capillary tip, *circa* 0.2 cc. of the liquid to be measured is now introduced into the body of the vessel without injuring the surface of the agar.   Occasionally, an air bubble may be trapped between liquid and plug but this can always be removed by sharp thumping with the finger. Then, holding the vessel at an angle of about 45°, place a pinch of quinhydrone powder inside it at the shoulder between B and C. With the aid of the platinum electrode mix the powder into a paste with a drop of the liquid from B and then mix thoroughly with the bulk of the liquid.   Immediately thereafter, support the electrode in the vessel by means of its cork and place the entire cell in the bridge.

Although an occasional drift in E.M.F. may be observed, a steady value will ordinarily be attained in 3 to 5 minutes and persist for at least 30 minutes thereafter.   In fact, it has been repeatedly observed that removal of the cell from the bridge and subsequent replacement will not affect its potential significantly, provided the plug be not injured in the process.   One cause for a drifting reading is an unclean platinum wire.   Treatment with hot concentrated nitric acid or cleaning mixture will not always be found satisfactory unless the liquid be heated for a long time.   However, gentle scrubbing with soap and a small stiff brush, followed by rinsing with distilled water, will be found entirely adequate. Another occasional source of difficulty may be encountered in the presence of minute traces of blood in the sample of gastric juice. In such cases, the shift in E.M.F. is usually accompanied by a gradual coloring of the liquid.   At first, all determinations were made in a thermostat at 25°.   Subsequently, because of the nature of the liquid under investigation as well as the slight fluctuations in temperature which occurred in a protected corner of the laboratory, it was decided to work at room temperature.

As a criterion of the reliability of the entire technique, it was found that forty-seven determinations on a standard 0.1 N HCl solution, made over a period of 4 months, gave an average value of 0.3901 volts ±0.00025.   Likewise, nineteen determinations on the same fluid, with cotton plugs instead of the KCl-agar, gave 0.3903 volts ±0.0003; sixteen determinations on a sample of gastric

juice gave an average deviation of ±0.00035 volts. In all of these experiments, the random use of point, spiral, or gauze electrodes indicated no significant differences in their behavior. Since the above average deviations in all cases are of the order of magnitude of 0.005 pH, and since the E.M.F. obtained with 0.1 N HCl is in close agreement with that given by Clark (1928),[3] the technique here described is entirely satisfactory.

To calculate the pH, the following formula from Cullen and Biilman (1925) was used.

$$pH = pH_{(0.1 \text{ N HCl})} + \left(\frac{E_{(0.1 \text{ N HCl})} - E_{(unknown)}}{0.0591} \right)$$

For the pH of 0.1 N HCl, Lewis and Randall's value of 1.08 was taken. Accepting the E.M.F. for this standard solution as 0.3901, the formula becomes

$$pH = 7.68 - \frac{E}{0.0591}$$

Micro-Titration Procedure

End-Points and Indicators—In view of the presence of some buffer substance in gastric juice, it was essential that the end-points be carefully controlled by comparison with buffered colorimetric standards. For free acidity pH 3.5 was chosen, since this is approximately the end-point given by Töpfer's reagent. When 1 cc. of a 0.1 N HCl solution is titrated in this way, only about one-half of 1 per cent remains unneutralized, since pH 3.5 corresponds to 0.0003 M. Consequently, this end-point is entirely adequate as a measure of "free" acidity. As indicator, Töpfer's reagent was replaced by brom-phenol blue because the latter shows a more easily discernible color change at pH 3.5 than does the former. For total acidity, the pH chosen was 7.0 to 7.2. Since phenol-phthalein, ordinarily used for this titration, was replaced by phenol red, it was desirable to show that pure gastric juice has a negligible buffer capacity between pH 7 and pH 8, *i.e.* that both the above indicators will give the same titer. In the case of stomach

[3] For the system: Pt, quinhydrone | 0.1 M HCl | saturated KCl |saturated KCl, HgCl | Hg,Pt the E.M.F. is given as 0.3904 volts at 20° and 0.3898 volts at 25° (p. 672).

contents, this is probably not so because of the complex nature of the ingested material. From Table II, however, it appears that in the fluid from stomach pouches there is practically no protein or other substances which bind the hydrochloric acid in this region of the pH scale. Several samples of gastric juice were titrated with 0.1 N alkali to pH 7.0, 8.0, and 10.0, with phenol red, phenolphthalein, and thymolphthalein respectively. The resultant values in Columns 2 and 3 of Table II are identical within experimental error. A comparison of Columns 3 and 4, however, indicates a small but significant buffer capacity between pH 8 and pH 10. The corresponding titration values for 0.1 N HCl are also given for purposes of comparison.

TABLE II

Buffering Power of Gastric Juice at pH 7 to pH 10

Sample No.	Cc. of 0.1 N NaOH per cc. of sample		
	pH 7	pH 8	pH 10
(1)	(2)	(3)	(4)
1	1.324	1.326	1.342
2	1.465	1.475	1.597
3	1.489	1.491	1.595
4	1.485	1.486	1.555
5	1.282	1.295	1.374
6	1.411	1.419	1.485
7	1.400	1.416	1.518
HCl 0.100 N	1.002	1.008	1.010

Final Volume of Titration Mixture—Because of the increase in pH of an unbuffered acid solution which results from dilution alone, it was necessary that the final volume be kept as low as possible. Otherwise, this dilution effect would introduce an appreciable error in the volume of alkali required to change the pH to that of the end-point for free acidity. In fact, it was found by actual calculation that a 5-fold dilution of the final mixture might introduce an error in the free and combined acidities of 3 mM. Consequently, the following precautions were taken. (1) No wash water was used throughout the titration. (2) Standard alkali of 0.1 N concentration was used with a micro burette, rather than a more dilute solution with a macro burette. This has the

added advantage of an increased sharpness of end-point. (3) Indicators were employed in such concentration that only 1 drop was necessary for each titration.

Titration Technique—The burette was graduated to read directly to 0.02 cc. In order to make estimations to 0.002 cc. possible with a fair degree of accuracy, the total length of burette between the zero and 10 cc. marks was about 60 cm. Instead of a tip drawn in the usual way, a Luer adapter was fused to the outlet end. Then, by means of a 22 gage stainless steel hypodermic needle, with the beveled point ground off entirely, it was possible to obtain freely falling drops of about 0.01 cc. However, with many of the samples of gastric juice titrated, the end-point was approached very rapidly because of the low buffer content. This necessitated the addition of reagent in very minute amounts (*i.e.*, fractional drops) near the end-point. With the aid of a thin cup-shaped stirring rod, it was possible to remove as little as 0.002 cc. of liquid from the tip, the amount being controlled by suitable diminution in the rate of drop formation. Irregularities in the removal of such minute quantities, due to spreading of the drop across the surface of the orifice, was entirely eliminated in consequence of the thin wall of the needle. The entire manipulation was greatly facilitated by the use of a mechanical drop control that has been described elsewhere (Hollander, 1931). The burette can be cleaned and filled most easily through the Luer adapter, with the application of very gentle suction at the top.

The titrations were performed in ordinary 10 cc. test-tubes. The liquid[4] to be titrated was delivered from a 0.5 cc. or a 1 cc. calibrated pipette, and 1 drop of indicator was added. Two similar tubes, each containing 2 cc. of an appropriate buffer solution, were also prepared. Thorough mixing of the titration mixture was effected by tilting and rotating the tube, as well as with the aid of the stirring rod. Addition of reagent was continued until the color of the solution was between those of the two reference tubes. Results of such determinations carried out on stand-

[4] Although Christiansen (1911) found that filtration was not necessary before titrating with various indicators, all specimens of juice used here were so treated immediately after collection. This was done in order to prevent digestion of any mucin present, the result of which would be an increase in the combined acidity value.

ard HCl solutions were in complete agreement with the values obtained by the macro method.

SUMMARY

The micro methods used in this series of studies for the determination of pH and titration acidities have been described.

It has been shown that the free acidity values of gastric juice from stomach pouch dogs, determined by titrating with bromphenol blue to a pH of 3.5, are in very close agreement with the pH values determined with the quinhydrone electrode.

The author wishes to express his indebtedness to Dr. David Kalkstein for the latter's assistance in part of this work.

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THE DURATION OF THE EFFECT OF WINTER SUNLIGHT ON BONE FORMATION IN THE CHICKEN*

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Goodale (1) has demonstrated a duration of the effect on growth in the chicken of the radiations from a quartz mercury lamp and Hart (2) and his associates have noted an improved calcium assimilation in baby chicks when as little as 3 minutes per week of these radiations are used. In a previous communication from this laboratory (3) we reported a duration of the effect of ultraviolet radiation from a quartz mercury lamp on bone formation in the young chick. The present report is concerned with the duration of the effect on bone formation when winter sunlight is used as the source of the ultra-violet radiation.

Experimental Procedure

The procedure was essentially that described in an earlier report (3). The ration fed throughout the life of the chicks consisted of 99 per cent yellow corn, 1 per cent sodium chloride and liquid skimmed milk, both foods being offered *ad libitum*. The exposures to sunlight were made in a house with a vertical south window of an ultra-violet transmitting material, Cel-O-Glass, 5.5 feet high and 9.5 feet wide. A canvas partition was placed so that the birds could not move more than 3 feet away from the base of the

window, thus insuring continuous incidence of the sun's rays during the period of exposure.

Lot 1 was exposed for 1 day, February 19, 1928, when the chicks were 11 days of age. It was a clear, cloudless day of bright sunshine.
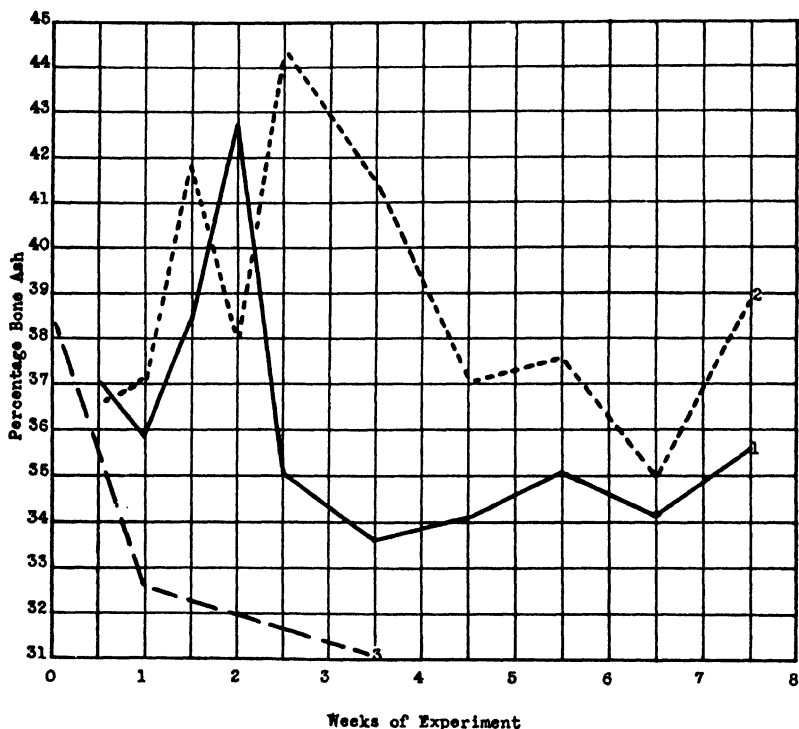
FIG. 1. Percentage of ash of thigh bones (femur). Curve 1 shows the effect of 1 day of exposure to sunlight and Curve 2 the effect of 3 days of exposure. Curve 3, no exposure.

Lot 2 was exposed with Lot 1 on February 19 and also on February 20 and 21. The same weather conditions prevailed on the 20th as on the 19th but the 21st was partly cloudy. Hence this lot was exposed during 2 bright days and 1 partly cloudy day.

Lot 3 was used as a control lot and was not given any exposure.

The only exposure of any of the lots to ultra-violet radiation was that described under Lots 1 and 2 above. Before the exposure period and afterward all lots were kept in diffused daylight which

had passed through window glass. After the exposure each lot was put into a separate pen. The analytical procedure was the same as that previously described (3), except that the ash determinations were made of pooled rather than individual bones.
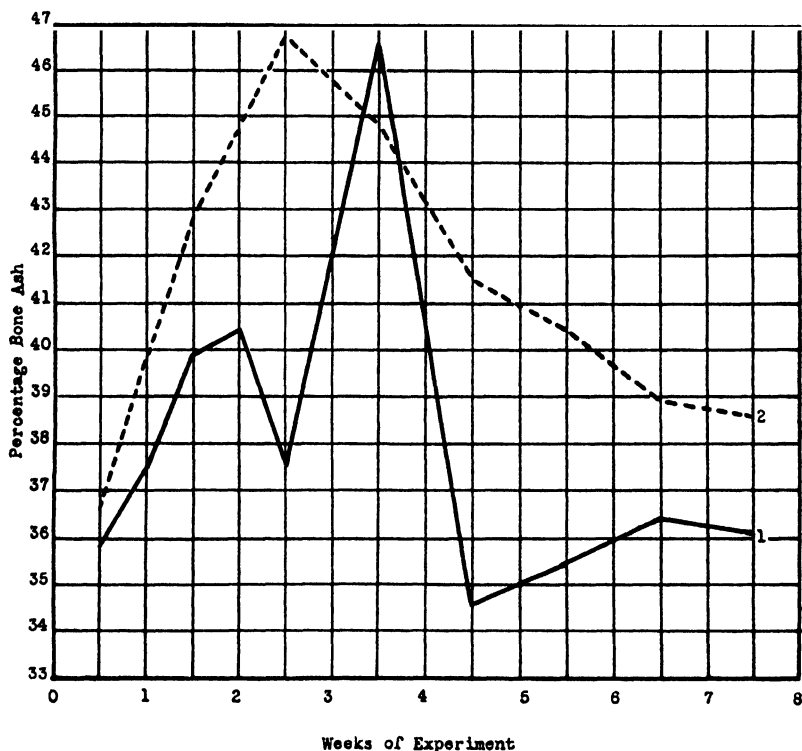
FIG. 2. Percentage of ash of wing bones (humerus). Curves 1 and 2 the same as in Fig. 1.

Results

Each point on the curves of Figs. 1 and 2 was determined by the percentage bone ash of the pooled bones of eight to ten individuals. The first sampling was made ½ week after the termination of the exposure of Lot 1 and a day and a half after the termination in the case of Lot 2, so that birds of the same age were selected. With the exception of Lot 3, groups were selected at half-weekly intervals for the first 2 weeks of the experiment and at weekly intervals thereafter until the completion of the study. The

basal group, Lot 3, was sampled three times during the experiment as indicated in Fig. 1.

It is obvious from the curves of Figs. 1 and 2 that there was a duration of the effect on bone formation of the exposures used. The trend of the curve of the group which received the 3 day radiation is generally higher indicating a more pronounced effect due to the longer exposure. In Lot 1 the appearance of leg weakness was delayed about 1 week and in Lot 2, 2½ or 3 weeks over its appearance in the basal group.

With the exception of the value for Lot 2 at 2 weeks and for Lot 1 at 3½ weeks of experiment, the trend of the wing bone curves is in agreement with that of the thigh bones. The marked fluctuations of the bone ash curves have been noted in an earlier report (3) and in unpublished data. The cause of the fluctuations is not known but they may be due in part to the analytical methods or to the method of sampling. The more frequent record of observations during the early weeks of the experiment probably contributes to the irregular appearance of the curves.

The low values obtained with the basal group are ample evidence that the conditions under which Lots 1 and 2 were kept after exposure to sunlight were not responsible for the higher bone ash values of these two lots.

SUMMARY

The exposure of young chicks to 1 day of winter sunlight transmitted through an ultra-violet transmitting material resulted in a duration of effect on bone formation and delayed the onset of leg weakness. A 3 day exposure caused a longer duration of effect on bone formation and delayed, for a still longer period, the appearance of leg weakness.

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ON THE MONOMETHYL GLUCOSE OF PACSU

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For an investigation now in progress in this laboratory, 4-methyl glucose was required, and as Pacsu[1] had prepared a substance to which he ascribed this structure, we undertook its preparation by his procedure. However, in a recent paper, Brigl and Schinle[2] describe 2-methyl glucose with physical properties practically identical with those given by Pacsu for his methyl glucose. Moreover, the 2-methyl-1,1-diethylmercapto-*d*-glucose of Brigl and Schinle is apparently identical with the methyl-1,1-diethylmercapto-*d*-glucose which resulted when we extended Pacsu's procedure to diethylmercaptoglucose. These considerations led us to subject the methyl glucose of Pacsu to more rigorous tests with the result that its identity with the 2-methyl-*d*-glucose of Brigl and Schinle has been definitely established.

The methyl glucose was prepared from 1,1-dibenzylmercapto-glucose according to the procedures of Pacsu. The analysis corresponded to a monomethyl hexose, and the melting point and rotations agreed with those reported by Pacsu, as well as with those reported by Brigl and Schinle.

On treatment with phenylhydrazine in methyl alcohol solution, this methyl glucose gave a phenylhydrazone which had the same properties as the corresponding derivative of the 2-methyl glucose of Brigl and Schinle. Moreover, like their 2-methyl glucose, on heating with excess phenylhydrazine in dilute acetic acid solution, it lost the methyl group and gave glucosazone, and not a methyl hexosazone, as reported by Pacsu.

[1] Pacsu, E., *Ber. chem. Ges.*, **58**, 1455 (1925).
[2] Brigl, P., and Schinle, R., *Ber. chem. Ges.*, **63**, 2884 (1930).

Additional proof of the identity of the methyl glucose of Pacsu and the 2-methyl-*d*-glucose of Brigl and Schinle was afforded by the fact that their 1,1-diethylmercapto derivatives are similar.

These facts would suffice to establish the identity of Pacsu's supposed 4-methyl glucose with the 2-methyl glucose of Brigl and Schinle. However, for further confirmation, the structure of the substance was arrived at by demonstrating that the hydroxyls in positions 3, 4, 5, and 6 were unsubstituted. Position 3 had already been excluded by Pacsu, as his methyl glucose was different from the 3-methyl glucose prepared from diacetone glucose. Position 6 is excluded by us as the methyl glucose of Pacsu oxidizes to a methyl saccharic acid. Positions 4 and 5 are excluded as changes in the optical rotation of a methyl alcohol solution of the sugar, containing 0.5 per cent hydrogen chloride, indicate both furanoside and pyranoside formation. Thus, the methyl group must be attached to the hydroxyl of atom 2.

In agreement with this view is the fact that on oxidation by the method of Lehmann-Maquenne,[3] the sugar consumes only 0.87 equivalents of oxygen per mol. This value is close to those found by Sobotka[4] for substances substituted in position 2 (2, 3-dimethyl glucose, 0.59; 2,3,4,6-tetramethyl glucose, 0.82) and quite different from those found when position 2 was unsubstituted (glucose, 5.16; 3-monomethyl glucose, 3.21; 3,5,6-trimethyl glucose, 1.98).

It may be questioned as to whether the monomethyl glucose of Pacsu is actually derived through a diacetone derivative, but if so then the latter must have position 2 open.

In addition, it may be mentioned that Pacsu claimed that the osazone of his methyl glucose was identical with that of methyl mannose prepared in a similar manner.[5] Granting this, it is warranted to conclude that in the methyl mannose of Pacsu, also, the methyl group is in position 2.

<div align="center">EXPERIMENTAL</div>

Methyl Glucose (*Pacsu*)—1,1-dibenzylmercaptoglucose (m.p. 137°) was acetonated and then methylated according to the pro-

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[3] See Griesbach, W., and Strassner, H., *Z. physiol. Chem.*, **88**, 199 (1913).

[4] Sobotka, H. H., *J. Biol. Chem.*, **69**, 267 (1926).

[5] Pacsu, E., and Kary, C., *Ber. chem. Ges.*, **62**, 2811 (1929).

cedure of Pacsu.[1]   After removing the acetone residues by hydrolysis, the resulting methyl 1,1-dibenzylmercaptoglucose melted at 194°.   The methyl glucose obtained after removal of the mercapto groups melted at 158°.   Its rotation, 3 minutes after dissolving was $[\alpha]_D^{23} = +21.1°$ in water, and the rotation at equilibrium was

$$[\alpha]_D^{25} = \frac{+1.21° \times 100}{2 \times 0.97} = +62.1°$$

The analysis corresponded to that of a methyl hexose.

4.395 mg. substance: 6.955 mg. CO_2 and 2.900 mg. H_2O.
0.1010 gm.   "   : 0.1150 gm. AgI.
$C_7H_{14}O_6$. Calculated. C 43.27, H 7.27, OCH_3 15.98
              Found.   " 43.15, " 7.38,   "   15.00

Pacsu reported a melting point of 156–157° and optical rotations of +18.57° after 3 minutes and +61.9° at equilibrium.   Brigl and Schinle[2] gave the melting point as 158° and the rotations +56.6° after one-half hour and +65.6° at equilibrium.

The reducing value of the methyl glucose was determined by the Lehmann-Maquenne[3] method and in the same manner as the determinations of Sobotka[4] so as to be comparable with his results. A sample of 3-methyl glucose was run similarly to afford a check on the technique used.

29.1 mg. of the above methyl glucose required 1.3 cc. of 0.1 N thiosulfate or 0.87 equivalents of oxygen per mol and 29.1 mg. of 3-methyl glucose required 4.7 cc. or 3.14 equivalents per mol. Sobotka found 3.21 equivalents per mol for 3-methyl glucose.

Phenylhydrazone—To 1.0 gm. of the methyl glucose in a small crystallizing dish there was added a solution of 0.56 gm. of phenylhydrazine (1 mol per mol of sugar) in about 10 cc. of methyl alcohol, and the mixture was warmed on the steam bath and stirred. The sugar dissolved, giving an almost colorless solution.   When nearly all the alcohol had been removed by evaporation, the dish was removed from the steam bath, and on further stirring the syrup crystallized as an almost solid mass.   The product was once recrystallized from 5 per cent ethyl alcohol and was then per-

fectly colorless. The yield of recrystallized product was 0.25 gm. The analysis corresponded to a phenylhydrazone of a methyl hexose.

4.020 mg. substance: 0.344 cc. N (757 mm. and 25°).
4.655 "   "   : 4.175 mg. AgI.
$C_{13}H_{20}O_5N_2$.   Calculated.   N 9.86, OCH_3 10.92
           Found.   " 9.76,   "   11.79

The melting point was 177–178° with no decomposition. The rotation, 3 minutes after dissolving was

$$[\alpha]_D^{25} = \frac{-0.36° \times 100}{2 \times 2.0} = -9.0° \text{ (in pyridine)}$$

The solution, which was originally colorless became rapidly yellow so that the end value (+8.0°) is probably not significant. Brigl and Schinle[2,6] report 177° and 178° as the melting points of the hydrazone of 2-methyl glucose and a specific rotation in pyridine of −12.3 to −13.3°. Hickinbottom[7] gave the melting point as 175–176° and Lieser[8] 176° (uncorrected).

Phenylosazone—To 2 gm. of the methyl glucose in about 100 cc. of water were added 3.4 gm. of phenylhydrazine (3 mols per mol of sugar) and 3.5 cc. of glacial acetic acid. The mixture was heated on the steam bath and the osazone soon began to crystallize. Its rate of formation was slower, however, than from an equivalent weight of glucose and although the mixture was heated for 1½ hours, the yield was much less than would have been expected from the same amount of glucose. The solution was cooled, the osazone filtered off and washed with methyl alcohol. It was then a felt of light canary-yellow needles. Yield 0.3 gm. The analysis corresponded to that of a hexosazone, a micro methoxy determination being entirely negative after heating for 2 hours.

4.106 mg. substance: 0.572 cc. N (757 mm. and 26°).
    $C_{18}H_{22}O_4N_4$.   Calculated.   N 15.64.   Found.   N 15.83

The melting point was 208–210° as was that of an intimate mixture with a sample of pure glucosazone. The rotation was

[6] Brigl, P., and Schinle, R., *Ber. chem. Ges.*, **62**, 1716 (1929).
[7] Hickinbottom, W. J., *J. Chem. Soc.*, 3140 (1928).
[8] Lieser, T., *Ann. Chem.*, **470**, 104 (1929).

$-78°$ after 15 minutes and $[\alpha]_D^{23} = \dfrac{-0.05° \times 100}{0.5 \times 0.33} = -30°$ at equilibrium (in pyridine-absolute alcohol, 2:3 by volume).

Pacsu described his osazone as melting at 198° and as having an initial rotation of $-50.3°$ and a final one of $-34.8°$. The analysis of his product corresponded quite well to that of a methyl hexosazone, but he made no methoxyl determination and the calculated analysis of a methyl hexosazone is not very different from that of the simple hexosazone.

$C_{18}H_{22}O_4N_4$. Calculated. C 60.31, H 6.19, N 15.64
$C_{19}H_{24}O_4N_4$. " " 61.25, " 6.48, " 15.06
Found (Pacsu). " 61.30, " 6.41, " 15.23

Methyl-1,1-Diethylmercaptoglucose—The procedures given by Pacsu were employed to obtain the methyl glucose from diethylmercaptoglucose instead of from dibenzylmercaptoglucose. The methyl-1,1-diethylmercaptoglucose obtained in this way melted at 157–158° and had a rotation of

$$[\alpha]_D^{23} = \frac{-1.51° \times 100}{2 \times 3.0} = -25.2° \text{ (in pyridine)}$$

The analysis was as follows:

4.810 mg. substance: 7.750 mg. CO_2 and 3.410 mg. H_2O.
3.655 " " : 5.667 " $BaSO_4$.
$C_{11}H_{24}O_5S_2$. Calculated. C 43.97, H 8.06, S 21.32
Found. " 43.93, " 7.93, " 21.30

Brigl and Schinle reported a rotation of $[\alpha]_D^{20} = -25.0°$ and a melting point[9] of 178°. Papadakis[10] gave melting points of 156–157° and 155°.

The same mercapto compound resulted on treating Pacsu's methyl sugar (prepared through the dibenzylmercaptoglucose) with ethyl mercaptan. 3.5 gm. of the methyl glucose were shaken with 3.5 cc. of concentrated hydrochloric acid (d 1.12) and 2.0

[9] As Brigl and Schinle state that their product is identical with that of Papadakis, and that mixed melting points show no depression, this value is perhaps due to a typographical error.

[10] Papadakis, P. E., *J. Am. Chem. Soc.*, **52**, 2147, 3465 (1930).

cc. of freshly distilled ethyl mercaptan until the mixture solidified, about 20 minutes. The solid was broken up and stirred with water, then filtered, and washed with water. The solid was recrystallized several times from 95 per cent alcohol. The product analyzed for a methyl diethylmercaptohexose.

4.959 mg. substance: 8.010 mg. CO_2 and 3.475 mg. H_2O.
5.144 " " : 7.973 " $BaSO_4$.
$C_{11}H_{24}O_5S_2$. Calculated. C 43.97, H 8.06, S 21.32
Found. " 44.04, " 7.84, " 21.29

The substance melted at 157° and its rotation was

$$[\alpha]_D^{25} = \frac{-1.01° \times 100}{2 \times 1.98} = -25.5° \text{ (in pyridine)}$$

Glucoside Formation—The glucoside formation in methyl alcohol containing 0.5 per cent of hydrogen chloride and 0.34 mols per

TABLE I
Observed Rotations during Glucoside Formation at 25°

Time		Rotation	Time		Rotation	Time	Rotation
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*degrees*
0	12	0.89	4	00	0.62	96	−0.12
0	30	0.84	7	30	0.36	172	−0.05
1	00	0.75	24	00	0.10	264	0.00
2	00	0.68	48	00	−0.06		

liter of the sugar was observed by following the changes in rotation of the solution. Experiments were made at room temperature (20–25°) and in baths of boiling chloroform and carbon tetrachloride. In the last two cases samples were sealed in glass tubes, cooled in ice, and after being in the bath for the assigned time, were again ice-cooled until the reading was to be made. .They were then quickly warmed to room temperature and read. In all cases the readings were made at 23–25° in a 2 dm. tube with sodium D light. The readings are given in Tables I, II, and III and the specific rotations calculated from them are plotted in Fig. 1.

Methyl Saccharic Acid.—2 gm. of the methyl glucose were oxidized

TABLE II

Observed Rotations during Glucoside Formation at 61°

Time		Rotation	Time		Rotation	Time		Rotation
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*min.*	*degrees*
0	00	0.99	1	15	−0.08	17	30	0.70
0	10	0.14	2	40	+0.08	27	00	0.92
0	20	0.03	5	00	0.25	38	00	1.01
0	40	−0.15	9	16	0.48	49	45	1.07

TABLE III

Observed Rotations during Glucoside Formation at 75°

Time		Rotation	Time		Rotation	Time		Rotation
hrs.	*min.*	*degrees*	*hrs.*	*min.*	*degrees*	*hrs.*	*min.*	*degrees*
0	00	0.99	0	50	0.15	10	00	0.95
0	05	0.07	1	30	0.34	19	30	1.16
0	10	−0.09	3	00	0.57	33	00	1.22
0	20	+0.01	5	00	0.74	49	45	1.23
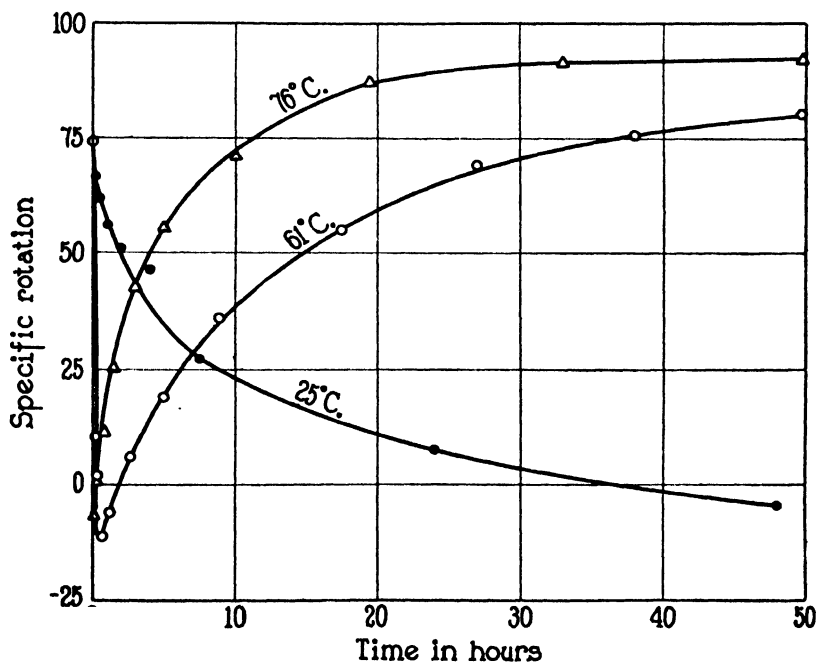
FIG. 1. Specific rotations during glucoside formation

with nitric acid as described by Levene and Meyer[11] for 3-methyl glucose.

After evaporating off all the nitric acid the residue was twice evaporated with water, then dissolved and converted to the calcium salt. This was purified by repeated precipitation from water with alcohol.

The analysis corresponded to the calcium salt of a methyl saccharic acid.

10.125 mg. substance: 5.215 mg. CaSO$_4$.
 9.820 "   " : 8.660 " AgI.
     C$_7$H$_{10}$O$_8$Ca. Calculated. Ca 15.28, OCH$_3$ 11.83
            Found.   " 14.99, "   11.64

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[11] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **54**, 805 (1922).

FURTHER INVESTIGATION OF QUANTITATIVE MEASUREMENT OF VITAMIN A VALUES*

BY H. C. SHERMAN AND E. L. BATCHELDER

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Early in the development of methods for the measurement of vitamin A values, workers in this laboratory, building upon the experience of Drummond, Coward, and their collaborators, directed their attention to the quantitative study of the relative growth response and physical vigor of test animals (rats) on different but definitely comparable levels of intake of vitamin A (or its precursor). For convenience, the term vitamin A will probably continue to be used as applying to the "factor" having the accepted nutritional significance, whether in any given case this factor be, chemically speaking, the substance as it actually functions in the animal, or the precursor which the animal receives from the plant, or a mixture of the two. At any rate the term "vitamin A value" may still be used in this broader sense even if the substance to which plants owe their vitamin A value is not the completely formed vitamin A but a precursor such as carotene.

From the point of view of our present conceptions, the work of Munsell (1) included two fairly extended series of experiments, one of which involved the feeding of butter fat, containing the vitamin itself, and the other the feeding of tomato, presumably owing its vitamin A value to the precursor. At that time, there was no reason for making such a distinction, and in the preparation of a very condensed journal article based upon these experiments (2) only the one more comprehensive set of curves was given—the one representing the results obtained by the feeding of graded

portions of tomato as sole source of vitamin A. The experiments with tomato were not only more comprehensive than those then made with milk as a source of vitamin A, but also their results were taken as being more conservative in that the fewer data of the milk feeding experiments showed a more accentuated response (which it was thought might be less regularly realized in the testing of foods in general) to increasing levels of feeding.

From the view-point of present day knowledge, however, it is readily conceivable that the less accentuated response to increasing levels of feeding of the tomato may have been because this was a less adequate source than milk of some factor or factors whose influence upon growth was not fully realized at the time of the earlier experiments or possibly that, notwithstanding the general equivalence of the vitamin A of animal origin and its precursor of vegetable origin, there may still be a difference in the degree of readiness and completeness of utilization of the two forms which might become measurable in the averages of sufficiently numerous experiments made in as rigorously quantitative a way as our present knowledge and experience permit.

For these reasons there are here placed on record the results of a more recent series of experiments in which the graded portions of vitamin A fed were given in the form of (dried) whole milk which undoubtedly contains the vitamin in the fully developed form, and under such conditions as to the basal ration used and the known nutritional backgrounds of the experimental animals used, as to take full account of all nutritional factors now recognized or even tentatively apprehended.

The general technique was that of Sherman and Burtis (3).

The basal diet here employed consisted of casein (thrice extracted with hot 95 per cent alcohol), 18 per cent; Osborne-Mendel (4) salt mixture, 4 per cent; sodium chloride, 1 per cent; dried brewers' yeast, 10 per cent; corn-starch (carrying vitamin D), 67 per cent.

Vitamin D was amply supplied by the incorporation of activated ergosterol (irradiated commercial cholesterol) into the corn-starch used in the basal diet, in addition to which it is known as the result of other work in this laboratory that the whole milk powder here fed as source of vitamin A contains significant amounts of vitamin D also (5) and that under the conditions obtaining in this

laboratory the experimental animals still have bodily stores of vitamin D at the end of the vitamin A depletion period (6). Thus there was triple insurance against any shortage of vitamin D in the vitamin A experiments reported in the present paper.
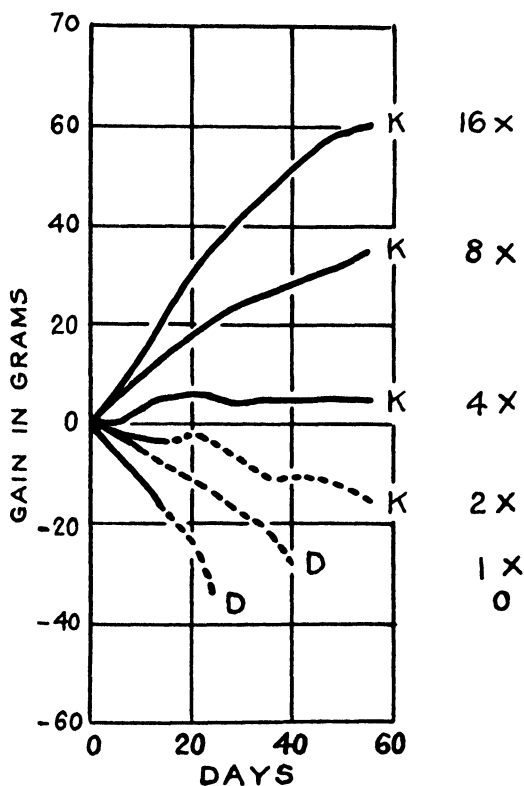
FIG. 1. Average gains of rats fed different amounts of vitamin A after having been depleted of their bodily store. Figures opposite end of each curve represent relative amounts of vitamin A fed. A broken line is used to indicate that one or more rats have died. When all animals died before the end of the 8 weeks period (*K*), the curve is terminated at a point representing the average weight and age at death (*D*).

Similarly the abundance of dried brewers' yeast used in the basal diet, the milk which served as source of vitamin A during the test period, and the bodily stores insured by the known nutritional backgrounds of the experimental animals, together seem ample to

insure adequate supplies of all of the less known nutritional factors essential for the growth of rats, with the possible exception of a slight shortage of the Coward factor (7) during the last week or two of the experimental periods of those animals (represented by the curve 16x in Fig. 1) which were growing at a much more rapid rate than that which we regularly employ in the measurement of vitamin A values.

The experimental animals here used were all from families which have been under continuous observation and control in this laboratory for many generations so that their nutritional and hereditary backgrounds are exceptionally well known. This has doubtless contributed to a somewhat higher degree of regularity in our results than could be expected if, with other conditions similar, the test animals had been purchased, or had come from newly established breeding lots, or had been bred in a stock colony of the ordinary type.

Young rats from our Diet B (a 1:2 mixture of dried whole milk and ground whole wheat plus table salt and distilled water)— supplemented in some cases with fresh lean beef—were separated from their mothers and placed on the basal diet at a uniform age of 4 weeks, and were then depleted of surplus bodily stores of vitamin A before the test periods or experimental periods proper were begun. Members of a given litter were distributed to the different levels of intake of vitamin A, and a total of nine test animals (four males and five females in each group except that of the negative controls) was fed at each level. Fig. 1 shows the average gain (or loss) during the test period for each of the six groups of nine animals, the group which served as negative controls, the group receiving the lowest fixed allowance of vitamin A, and four other groups each receiving twice as much vitamin A as the one next below it. The average weights were between 43 and 45 gm. at the beginning of the depletion period and between 90 and 94 gm. at the beginning of the test period.

It seems unnecessary to describe here the care used in insuring that all of the animals which were being fed in comparison with each other were subjected to uniform environmental conditions in every respect except for the quantitatively varied food factor under investigation, in the cleaning and sterilization of cages, and in the purification of the casein of the basal diet. The experience of

Osborne and Mendel (8) as well as of several workers in our own laboratory tends to show that such yeast as we have used does not contribute any significant amount of vitamin A. (Moreover the yeast is a constant factor in the diet of all the test animals including the negative controls.)

The negative controls of the present experiments may be regarded as test animals of known good heredity living under environmental conditions excellent in all (known) respects except for a practically complete lack of vitamin A. From the time that their depletion periods were ended (after they had definitely ceased to grow on the basal diet) and the test period of each was begun, they survived from 16 to 37 days, average 23.6 ± 1.4 days.

The nine animals parallel with these but receiving the smallest allowance of vitamin A here fed—only about one-fourth the quantity required for mere maintenance, and less than one-sixteenth the amount required for normal growth—showed greater individual variation, their survival periods ranging from 11 to 72 days. The average time of survival, however, exceeded that of the negative controls by 16.8 ± 3.9 days. As this difference is well over 4 times its probable error, we may conclude that, while individual results are even more variable at such extremely low levels of vitamin A intake than with the negative controls, yet the average of nine carefully controlled quantitative experiments sufficed to establish the difference due to this small amount of the vitamin, with a satisfactory degree of certainty. Evidently the method as now developed is capable of furnishing convincing evidence of the presence and approximate relative amount of even smaller amounts of the vitamin than we have previously supposed if the experiments are sufficiently numerous and sufficiently well controlled; but even the most rigorous quantitative control will not insure against misleading results if too few animals are used at these low levels. Animals receiving so little of the vitamin as to be losing weight must be regarded as definitely ill and cannot be expected to show as much regularity as is shown at the higher levels of feeding which induce steady (even if subnormal) gains in weight.

In the present series the animals fed at the "8x" level gained at slightly more than the average rate of 3 gm. per week, which previous experience has indicated as a desirable basis for quantitative comparisons. The average for this 8x level was 30.3 ± 3.5

gm. for the 8 weeks above that resulting from feeding half as much of the vitamin; and it was 24.9 ± 4.9 gm. (for the 8 weeks) below that resulting from the feeding of twice as much. These numerical differences, considered in relation to their probable errors, are plainly much more than sufficient to demonstrate a true difference of vitamin A intake. Inasmuch as we are here dealing with phenomena of the sort to which according to Rietz and Mitchell (9) the usual rules of probable error should apply, it is logical to consider, by means of interpolation, what degree of convincingness of findings may be expected from smaller differences of level of vitamin A feeding in the same general zone; *i.e.*, of adequacy to support an average rate of gain not too far from 3 gm. per week. Thus the interpolated result for the end of an 8 weeks test period on a level of feeding of 6x would be 20.1 ± 2.4 gm. and would differ from the 8x result by 15.1 ± 3.5 gm., which again is more than sufficient for ordinary purposes; for we can say that with the 8x level as a starting point, a reduction of 25 per cent in the amount of vitamin A actually present in the test food would have been demonstrated with a degree of certainty better than odds of 150:1. Again, the interpolated value for 7x would be 27.7 ± 2.4 gm., differing from the 8x result by 7.5 ± 3.5, corresponding to odds of about 10:1 which would not be acceptable for the definite establishment of this diminution of 12 per cent in the vitamin value of the 8x level of feeding.

Hence it appears that, with the method here used, with nine well standardized test animals on each level of feeding, and with the employment of the same degree of experimental facility and experience as here obtained, the minimum difference to be safely regarded as measurable would lie between 12 and 25 per cent, closer measurement becoming possible, according to well known statistical principles, by increasing the number of exactly comparable cases—while, on the other hand, averages of still more numerous data may be of much less value if as a matter of fact the actual conditions of the experiments were less strictly comparable. ·

If our estimates of attainable accuracy seem more optimistic than those of Drummond and Morton (10) or of Irwin, Brandt, and Nelson (11) it is probably because our animals were more perfectly matched as to litter, size, sex, and hereditary and nutritional backgrounds, and because we have here used a single food source of vitamin A, whereas in some cases (11) unknown variations

in the actual vitamin A values of the different materials fed may conceivably account for a large part of the variability of growth response reported.   In this connection Coward, Key, Morgan, and Cambden (12) have recently discussed the importance of a definite standard of reference in consideration of the accuracy of results. While employing a different basis of expression for our findings, we have sought to utilize the results of their experience in increasing and safeguarding the quantitative accuracy of our experimentation.

SUMMARY

Using, for the measurement of vitamin A values, the general method described in previous papers from this laboratory, with all added precautions of technique and interpretation suggested by recent advances in our knowledge of the vitamins, we have found that graded increments in the amounts of this vitamin, fed in the form of whole milk powder, cause increases in survival (at low levels of feeding) and in rate of gain of weight (at higher levels) which are relatively greater than have been shown in previously published work.   Simple statistical analysis of the experimental results indicates that, at levels of feeding which induce a gain in weight of about 3 gm. per week, a decrease of 25 per cent (or an increase of 33 per cent) is undoubtedly measurable by the method here used when nine or more carefully standardized experimental animals are employed at each level of feeding.

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TRYPTOPHANE METABOLISM

I. THE PRODUCTION OF KYNURENIC ACID FROM TRYPTOPHANE DERIVATIVES*

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Recent work (Berg, Rose, and Marvel, 1929–30) has shown that the essential amino acid, tryptophane, may be rendered unfit for supporting growth, either by benzoylation or by condensation with formaldehyde. The formaldehyde condensation product has been employed also by Jackson (1929) with a similar result. On the other hand, neither esterification with ethyl alcohol nor acetylation caused any detectable decrease in its growth-promoting ability. Although the ethyl ester hydrochloride used was shown to be very readily split by enzyme extracts, the acetyl derivative appeared to undergo cleavage only very slowly and incompletely.

Inasmuch as tryptophane undergoes a series of reactions in certain animal organisms by means of which it is converted into the quinoline derivative, kynurenic acid (Ellinger, 1904), it seemed of interest to employ the output of the latter product in the urine, following the administration of the tryptophane derivatives mentioned above, as a means of obtaining evidence regarding their cleavage *in vivo*. If the derivative in question should undergo enzymolysis either in the alimentary tract or in the body proper, the tryptophane liberated would cause an increase in urinary kynurenic acid elimination.

For the purpose of demonstrating the production of kynurenic acid after tryptophane administration, the dog and the rabbit are

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commonly used as experimental animals.   No species has been found, however, in which the conversion of the amino acid into the quinoline derivative is even approximately quantitative. Since kynurenic acid itself, administered to dogs or to rabbits, has been shown to be largely recoverable in the urine (Homer, 1915; Matsuoka, 1918), it seems likely that the explanation lies in an incomplete synthesis of this quinoline derivative in the body rather than in its partial destruction.   In experiments involving tryptophane administration in constant amount to dogs, Kotake and Ichihara (1927) have reported that difference in output of kynurenic acid in the urine may be ascribed to a variable partial excretion of the product in the bile.   Matsuoka (1918), however, showed that if the dietary regimen of rabbits is kept constant and a like amount of the amino acid is administered, the urinary output of kynurenic acid, especially in the same individual, does not vary greatly.   Consequently, although one cannot be too positive concerning the quantitative aspects of kynurenic acid output in the urine, he may, under constant conditions, expect fair comparisons.

<div align="center">EXPERIMENTAL</div>

The acetyl and benzoyl derivatives of tryptophane and the ethyl ester hydrochloride were prepared as directed by Berg, Rose, and Marvel (1929–30).   The formaldehyde condensation product was made according to the method of Homer (1913).   Each of the derivatives showed a melting point in close agreement with that found by the former workers.   No deviation from theoretical was obtained when portions of each derivative were mixed with samples of the same product prepared by Berg, Rose, and Marvel.

Male rabbits were employed as experimental animals.   They were kept in individual metabolism cages and given water and oats ad libitum and limited daily allotments of alfalfa hay and carrots. Urine samples were collected at 24 hour intervals, the portion not voided voluntarily being obtained at the end of each period by pressure on the abdomen.   Each 24 hour specimen was analyzed quantitatively for kynurenic acid by the Capaldi (1897) isolation method, which follows:

The urine sample is treated with half its volume of 10 per cent barium chloride solution containing 5 per cent ammonium hy-

droxide, and is allowed to stand half an hour before filtering. The filtrate is evaporated on a steam bath to one-third the volume occupied by the original urine sample, filtered if necessary, and made strongly acid to Congo red with 4 per cent hydrochloric acid. A precipitation occurs which is completed by allowing the solution to stand in the ice box 16 to 24 hours. The precipitate is filtered off, washed with 1 per cent hydrochloric acid, and dissolved in ammonium hydroxide. The ammoniacal solution is filtered, heated on the steam bath to drive off the excess ammonia and to reduce it to a small volume, and finally made acid with 4 per cent hydrochloric acid. After standing in the ice box at least 6 hours, the precipitate is filtered off on a weighed Gooch crucible, washed once with 1 per cent hydrochloric acid, twice with distilled water, and dried in an oven at 110° to constant weight.

In carrying out the above procedure on urine collected after no tryptophane was administered, it was noticed that invariably a small amount of flocculent precipitate was obtained. It was deemed best, therefore, to subject each 24 hour sample, not only on experimental, but also on intervening, days to analysis. Furthermore, an interval of 2 days was usually allowed to elapse between the 24 hour periods in which tryptophane or the tryptophane derivative was administered.

The kynurenic acid precipitate obtained after the administration of tryptophane was found to melt around 260° (uncorrected). The product isolated is brown in color and forms a deeply pigmented solution. Upon dissolving the crude substance in ammonium hydroxide, evaporating off the excess ammonia, and acidifying carefully with acetic acid, a dark flocculent material separates out. The precipitate is filtered off and the filtrate, upon being boiled twice with bone-black, becomes lemon-yellow in color and yields, upon further acidification with 4 per cent hydrochloric acid, ·a cream-colored product which, when filtered, washed, and dried at 110°, melts at 275° (uncorrected). Because kynurenic acid is extremely difficult to purify, widely varying melting points have been recorded in the literature (264-266°, Schmiedeberg and Schultzen (1872); 257-258°, Kretschy (1881); 288-289°, Homer (1914)). Miss Homer's data show that her Capaldi precipitates became sticky at 260°. After a partial purification similar to the one used in these studies, the melting point

was raised to 278°.   The 288–289° value was obtained only after employing a series of intensive and wasteful purification steps.

In order to test the method of isolation, rabbit urine which contained addition of the partially purified kynurenic acid or which was collected during the 24 hours following the subcutaneous administration of the product to the animal, was subjected to the Capaldi procedure.   Results are shown in Table I.

In the first two cases (in Table I) the net recovery was determined by subtracting the weight of the material obtained in a blank determination on one-half of the urine from the weight of the precipitate isolated after kynurenic acid addition to the other half.   In the latter cases the precipitate from the 24 hour urine sample collected previous to the experiment was used as the blank.

TABLE I

Kynurenic Acid Recoveries by the Capaldi Procedure

Weight of partially purified kynurenic acid used	Net weight of kynurenic acid isolated	Recovery
gm.	*gm.*	*per cent*
0.1500 added	0.1438	95.9
0.1500 "	0.1419	94.6
0.1500 injected subcutaneously	0.1386	92.4
0.1500 " "	0.1200	80.0
0.3000 " "	0.2755	91.8
0.3000 " "	0.2528	84.3

The data obtained show that the procedure is capable of giving good results and that kynurenic acid, injected into rabbits subcutaneously, may be recovered satisfactorily from the urine.

The acetyl, benzoyl, methylene,[1] and ethyl ester hydrochloride derivatives of tryptophane were administered, as was the amino acid itself, both subcutaneously and by mouth, in amounts equivalent to 1 gm. of tryptophane.   Results of the series of experiments are incorporated in Tables II to IV.

On days other than those following the administration of products to be tested for kynurenic acid production, scanty amor-

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[1] It has been assumed that the formaldehyde condensation product is a hydrated methylenetryptophane (*cf.* Homer, 1913), and the terms have, therefore, been used interchangeably in this paper.

TABLE II

Kynurenic Acid Elimination Following Subcutaneous Administration of Tryptophane or Tryptophane Derivatives

Day	Apparent kynurenic acid precipitate		1 gm. tryptophane (in 2 equal doses, 6 hrs. apart) in form of
	Rabbit 1, male, 3 kilos	Rabbit 2, male, 3 kilos	
	gm.	*gm.*	
1-3	0.0068	0.0087	
4	0.2290	0.1752	Sodium salt
5-6	0.0047	0.0011	
7	0.2094	0.3241	Hydrochloride
8-9	0.0033	0.0066	
10	0.3040	0.2598	Ethyl ester hydrochloride
11-12	0.0037	0.0496*	
13	0.0097	0.0461†	Sodium salt of acetyl derivative
14-15	0.0052	0.0050	
16	0.0473‡	0.0471‡	Sodium salt of benzoyl derivative
17-18	0.0058	0.0152	
19	0.0015	0.0116	Methylene derivative in water suspension
20-21	0.0037	0.0065	
22	0.1977	0.3056	Water solution
23-24	0.0041	0.0067	

The values for days 1 to 3, etc., are the averages per day.

* This high average value was due to an increased excretion on the 11th day, probably accounted for by failure to drain the bladder completely in the previous period.

† The precipitate melted at 246-248° and gave a strongly positive Hopkins-Cole test; when washed with 5 cc. of butyl alcohol saturated with water, a residue of 0.0270 gm. was obtained which gave a negative Hopkins-Cole test and melted at 263-264°.

‡ The precipitates melted at 99-107° and 108-129° respectively, and responded strongly to the Hopkins-Cole test; when washed with 5 cc. portions of water-saturated butyl alcohol, residues of 0.0002 and 0.0024 gm., respectively, remained.

phous precipitates were obtained by the Capaldi procedure. In-
asmuch as these precipitates were very small and very highly
pigmented, tests applied to demonstrate the presence of kynurenic

TABLE III

*Kynurenic Acid Elimination Following Administration of Tryptophane or
Acetyltryptophane by Stomach Tube*

Day	Apparent kynurenic acid precipitate		1 gm. tryptophane in form of
	Rabbit 3, male, 2.4 kilos	Rabbit 4, male, 3.15 kilos	
	gm.	*gm.*	
1–2	0.0146		
3	0.0718*		Sodium salt of acetyl derivative
4–6	0.0127	0.0120	
7	0.0490†	0.0991†	" " " " "
8	0.0102	0.0213	
9	0.2137	0.1001	" "
10–11	0.0118	0.0079	
12	0.3368	0.3354	" "
13–15	0.0096	0.0087	
16	0.0308	0.0102	" " of acetyl derivative
17–18	0.0144	0.0017	
19	0.0214	0.0048	" " " " "
20–21	0.0125	0.0062	
22	0.2620	0.3150	" "

The values for days 1 to 2, etc., are the averages per day.
  * The residue, after being washed with 5 cc. of butyl alcohol, weighed
0.0391 gm.
  † The residues, after being washed with 5 cc. of butyl alcohol, weighed
0.0147 and 0.0653 gm., respectively.

acid were unsatisfactory. The diet was low in tryptophane but
not entirely free from it. The weights of such precipitates,
therefore, serve as blanks.

Following the administration of 1 gm. of tryptophane to the

various animals used, the urinary kynurenic acid elimination was found to range from 0.1001 to 0.3368 gm., with an average output for the twelve administrations of 0.2596 gm.

TABLE IV

Kynurenic Acid Elimination Following Administration Subcutaneously or per Os of Tryptophane, Tryptophane Derivatives, or Kynurenic Acid

Day	Kynurenic acid precipitate after washing with 5 cc. butyl alcohol		Substance administered
	Rabbit 7, male, 2.4 kilos	Rabbit 8, male, 2.5 kilos	
	gm.	*gm.*	
1-2	0.0092	0.0041	
3	0.3114	0.3270	1 gm. tryptophane subcutaneously (in 1 dose) as sodium salt
4-5	0.0119	0.0082	
6	0.2104*	0.1943*	1 gm. tryptophane subcutaneously (in 2 doses) as sodium salt of acetyl derivative
7-8	0.0041	0.0027	
9	0.0751	0.0757	1 gm. tryptophane *per os* (in 2 doses) as sodium salt of acetyl derivative ·
10-11	0.0039	0.0080	
12	0.0058	0.0029	1 gm. tryptophane *per os* (in 2 doses) as water suspension of methylene derivative
13-15	0.0088	0.0049	
16	0.2764	0.2486	0.3 gm. partially purified kynurenic acid (in 1 dose) subcutaneously as sodium salt
17-18	0.0048	0.0059	
19	0.0161	0.0121	1 gm. tryptophane *per os* (in 2 doses) as sodium salt of benzoyl derivative
20-22	0.0089	0.0084	
23	0.1300	0.1216	0.15 gm. of partially purified kynurenic acid (in 1 dose) subcutaneously as sodium salt

The values for days 1 to 2, etc., are the averages per day.

* The residues gave a positive Hopkins-Cole test and had a low melting point. Washing with an additional 5 cc. portion of butyl alcohol removed all but a trace of the indole body and raised the melting point to 258–261°. The residues weighed 0.1157 and 0.1423 gm., respectively.

Tryptophane ethyl ester hydrochloride, when injected subcu-
taneously in amounts equivalent to 1 gm. of the amino acid, yielded
0.2598 and 0.3040 gm. in the two trials made, an amount quite as
large as that obtained upon administering tryptophane itself. The
product isolated gave a good Jaffe test (1882–83), melted at the
same point as did the kynurenic acid samples obtained following
tryptophane administration, and showed no change in the melting
point upon being admixed with the latter.   Evidently the ethyl
ester hydrochloride derivative undergoes very ready cleavage in
the body to yield the free amino acid.

Methylenetryptophane, on the other hand, administered either
subcutaneously or *per os*, did not cause an increase in the weight
of the Capaldi precipitate over that found in the control periods.
The urine samples gave a strongly positive Hopkins-Cole reaction,
indicating that the compound could not have been completely
burned.

The weights of the precipitates obtained following the adminis-
tration of the benzoyl and acetyl derivatives might cause one to
assume that these undergo conversion into kynurenic acid.   How-
ever, the precipitates in these cases showed melting points well
below those obtained on the crude kynurenic acid isolated after
free tryptophane administration.   They also responded strongly
to the Hopkins-Cole test, suggesting contamination with some
indole product, if not the originally administered tryptophane
derivative itself.   Upon adding benzoyltryptophane and acetyl-
tryptophane to samples of rabbit urine and applying the Capaldi
isolation procedure, both were found to be partially recoverable.
However, the yield of each derivative obtained seemed to vary with
the individual urine to which it had been added, thus making the
method unreliable as a means of estimating recoveries of these
substances.   The possible excretion of the unchanged derivatives
following their administration to the animal would, therefore, also
interfere with the kynurenic acid estimation.   It was found, how-
ever, that butyl alcohol saturated with water (used by Dakin
(1918) in the preparation of tryptophane) was effective as a sol-
vent for the tryptophane derivatives, but fortunately not for ky-
nurenic acid.   In most cases, 5 cc. of the butyl alcohol reagent,
when triturated for 5 minutes with the precipitates isolated after
acetyltryptophane or benzoyltryptophane administration, were

effective in dissolving out completely the substances responding to the Hopkins-Cole test. Under similar conditions only 0.0035 gm. of partially purified kynurenic acid went into solution.

Application of this method to the Capaldi precipitates, following benzoyltryptophane administration subcutaneously, showed that all but an insignificantly small proportion of the precipitate was removed. Even with allowance for the possible loss of kynurenic acid by extraction, the weight of the residue was well within the control range. The Capaldi precipitates, in these instances, therefore, contained little, if any, kynurenic acid. It seems that benzoyltryptophane, injected subcutaneously, is not converted into kynurenic acid. The inability of the body to split this tryptophane derivative is in accord with the findings of Magnus-Levy (1907) that a number of benzoylated amino acids injected subcutaneously could, in practically every case, be recovered unchanged in the urine in an amount large enough to warrant the conclusion that they had not undergone cleavage; it is also in harmony with the observation of Lewis, Updegraff, and McGinty (1924) that the benzoylation of cystine effectively prevents its normal oxidation following subcutaneous injection of the product.

The results following administration of benzoyltryptophane *per os* are recorded in Table IV. For economy of space, only the weights of the precipitates after trituration with butyl alcohol are given. The original unextracted precipitates on the experimental day, as well as on the day following, were greater in amount than those isolated after the subcutaneous injection of this derivative. They responded to the Hopkins-Cole test and, with one exception, melted below 100° (benzoyltryptophane melts at 104–105°). The high melting point (152–172°) of one of the precipitates would seem to indicate considerable admixture of some other substance with the presumably recovered benzoyltryptophane. Inasmuch as all but a small portion of this precipitate was removed by washing with the 5 cc. of butyl alcohol, the admixed material was very soluble in that reagent. In view of the work done on benzoylcystine feeding by Lewis, Updegraff, and McGinty (1924), hippuric acid was suspected. Analyses showed that 0.2035 gm. of the latter product was dissolved by 5 cc. of the butyl alcohol during the usual 5 minute trituration.

Upon washing the Capaldi precipitates with butyl alcohol, ex-

tracting the washings with normal sodium hydroxide, acidifying the extract, and allowing it to stand in the ice box, a small precipitate separated which melted at 163–172° and gave a mixed melting point of 125–135° with benzoyltryptophane and 178–181° with hippuric acid (m.p. 187.5°). The product obtained gave a positive, though not intense, test for the indole ring, but was not isolated in quantity sufficient for purification or for use in the preparation of a derivative. The ready solubility of hippuric acid in the butyl alcohol, and the intermediate melting point obtained upon mixing hippuric acid with the material isolated from the Capaldi precipitate washings, tend to favor the assumption that hippuric acid is present, but these are not to be considered as conclusive evidence. The residues remaining after extraction of the original precipitates were not materially greater than those of some of the control periods. Certainly benzoyltryptophane administered *per os* does not yield an appreciable amount of kynurenic acid.

Although most of the apparent kynurenic acid precipitates isolated after administering acetyltryptophane contained a considerable admixture of material responding to the Hopkins-Cole test, the residue obtained after washing out the indole body usually melted at 260–262°, showed no depression from this point when mixed with crude kynurenic acid, and responded to the Jaffe test. In a few instances, 5 cc. of the butyl alcohol reagent were insufficient to rid the precipitate completely of the indole body; an additional 5 cc., however, were adequate. In view of the fact that acetyltryptophane answers as well in growth experiments as does tryptophane itself, one might expect fairly comparable conversions of the two into kynurenic acid. Such, however, appears not to be the case. The highest yield ever obtained after administration of the acetyl derivative was 0.1423 gm. (with allowance for a possible 0.0070 gm. extracted, 0.1493 gm. of kynurenic acid may have been present), a figure well below the average 0.2596 gm. obtained after tryptophane administration. The average amount of kynurenic acid produced from the several acetyltryptophane administrations, with allowance for the weight of the quinoline derivative extracted by the butyl alcohol, was 0.0516 gm. The butyl alcohol washings from a few of the more

copious precipitates were extracted with normal sodium hydroxide, made almost neutral with dilute acetic acid, boiled with bone-black, filtered, acidified strongly with sulfuric acid, and placed in the ice box overnight. Buff-colored crystals were obtained which gave an intense color with the Hopkins-Cole reagent, melted at 193-196°, and gave a mixed melting point of 201-203° with acetyltryptophane (m.p. 206-207°) and a neutral equivalent of 240 (theoretical for the acetyl derivative, 246.1). Upon recrystallization of the isolated product from alcohol, the melting point was raised to 202-204° (uncorrected). The administered acetyltryptophane seems, therefore, to be the indole body which is admixed with kynurenic acid in the precipitates isolated in the Capaldi procedure. Attention is called to the fact that acetyltryptophane given *per os* apparently does not cause a greater output of kynurenic acid than when it is injected subcutaneously. If the enzymes of the alimentary tract were able to hydrolyze the derivative readily, a significant increase in kynurenic acid output would result and recovery of the unaltered acetyltryptophane in the urine would not be likely.

Numerous examples of acetylation in the animal body have been recorded in the literature since the classical experiments of Knoop (1910, 1911). On the basis of work which showed that acetyltryptophane could be readily utilized for growth by the rat, despite the fact that appreciable enzymatic splitting *in vitro* could not be demonstrated, Berg, Rose, and Marvel (1929-30) suggested that the derivative underwent postabsorptive cleavage, thus affording "an interesting illustration of reversal of the reaction of biological acetylation. . . . " Data recorded in this paper confirm their assumption that deacetylation of the derivative does occur after absorption. Inasmuch as acetylation appears to be a mode of detoxication, it seems logical to assume that the reverse process might occur to an extent not far beyond that biologically expedient. Acetyltryptophane administered in small amounts is likely hydrolyzed in sufficient quantity to furnish adequate tryptophane for good growth, whereas an excess is probably excreted largely unchanged. The results of the feeding experiments and of the kynurenic acid studies may be explained equally as well on the basis of a limited enzymatic mechanism for deacetylation.

SUMMARY

The production of kynurenic acid from tryptophane derivatives has been used as a means of determining their cleavage *in vivo*. Of the four compounds employed, the ethyl ester hydrochloride alone yielded a quantity of kynurenic acid as great as that obtained after the administration of an equivalent amount of free tryptophane. The comparable yields after subcutaneous injection are evidence that the compound is readily split in the body. Neither methylenetryptophane nor benzoyltryptophane administration caused a significant increase in the output of kynurenic acid. Evidently tryptophane cannot be liberated in the body from either compound. There appeared to be no appreciable alimentary hydrolysis after administration of the derivatives *per os*.

Acetyltryptophane, though quite as good for growth promotion as the free tryptophane, is not readily convertible into kynurenic acid either after administration subcutaneously or *per os*. Hence, enzymolysis of the compound is not easily effected either in the alimentary tract or in the body. However, the production of definite, even though small, amounts of kynurenic acid from subcutaneously administered acetyltryptophane constitutes evidence that biological deacetylation can occur in the animal organism.

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THE METABOLIC RATE AND RESPIRATORY QUOTIENTS
OF RATS ON A FAT-DEFICIENT DIET

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In 1929 and 1930, Burr and Burr (1, 2) described a deficiency disease produced in rats by a fat-deficient, but otherwise balanced diet. This disease is superficially characterized by an arrest of growth, emaciation, scaliness of the feet, tail, and skin, excessive intake of water, hemorrhagic and necrotic tail, prolapse of the penis, bloody urine, and death of the animal. A careful study by Jackson has shown that the organs of these rats are practically free from fatty tissue. He has also described a characteristic degeneration of the kidneys (3). The disease is characterized by the fact that apparently complete cures are accomplished by relatively small amounts of the highly unsaturated acids, linolic and probably linolenic acids.

It was desired to determine the respiratory quotients and metabolic rate of these animals as part of a larger study of the metabolic and structural changes produced in the rats by the fat-deficient diet. In this connection, the work described in this paper attempts to ascertain whether or not animals on the fat-deficient diet are able to form fat from carbohydrate and, if so, whether fat is formed when a limited amount of carbohydrate is fed in the fasting condition, in which case, it is an abnormal metabolism of carbohydrate. It was also desired to ascertain whether or not the carbohydrate metabolism of these rats is affected by linolic acid which cures the deficiency disease, and by liver lipoids which have been shown by Wesson (4) to restore an apparently abnormal carbohydrate metabolism to normal. It was desired, as well, to learn whether the metabolic rate, both basal and carbohydrate assimilatory, is normal or abnormal in the diseased and cured rats.

In order to accomplish this, carbohydrate was fed to rats in various stages of the disease before and after curing, and the metabolic rate and respiratory quotients obtained following this carbohydrate test meal were compared with those obtained by Wesson (5) using the same procedure with normal rats. As nearly as possible the same uniform conditions of temperature, available metabolites, and previous environment were obtained. The animals, maintained since weaning at a temperature of 26–27° were, after 14 hours of fasting, given the calculated amount of dextrin required for 12 hours of normal metabolism, and the respiratory quotients and metabolic rate determined at 28° at approximately hourly periods for 12 hours following. They were then returned to their accustomed diet for 5 to 10 days before another series of determinations.

Diets

At weaning the rats were placed on a basic diet consisting of sucrose, purified casein, and 3.8 per cent salts (McCollum's Salt Mixture 185). Daily doses of 0.66 gm. of ether-extracted yeast and the unsaponifiable material from 72 mg. of cod liver oil (Patch), and from 36 mg. of wheat germ oil were added to the diet.

The drinking water contained 0.27 mg. of KI per liter, and the mixed diet about 0.1 mg. of KI per kilo. It being assumed that the average rat eats 10 gm. of food and drinks 20 cc. of water per day its daily KI intake would be about 0.006 mg. of KI. This amount of iodine is not far from an ordinary normal intake. The fat content of the diet was between 0.01 and 0.1 per cents.

The rats that were used in this series may be grouped as follows:

Group A, High Carbohydrate Diet—Sucrose 84.2 per cent, casein 12 per cent, and salts 3.8 per cent were supplemented by vitamins A, B, D, and E, as outlined in the preceding paragraph.

Group B, "Treated"—These rats, maintained on the high carbohydrate diet described under Group A, were given daily 3 drops of a mixture of equal parts of methyl linolate and methyl linolenate when symptoms of the fat-free disease had become pronounced. The curative unsaturated acids were fed from April 29 to August 4, and then discontinued. At the time the respiratory determinations were made (October and November) the animals were slowly reverting to the diseased condition.

Group C, High Protein Diet—Sucrose 31.2 per cent, casein 65 per cent, and salts 3.8 per cent were supplemented by vitamins A, B, D, and E as for Group A.   One rat (Rat W 30036) of this group was reared on a casein, salt, and vitamin mixture containing no sucrose.

Group D, Tung Oil with High Carbohydrate Diet—5 drops of tung oil daily were added to the high carbohydrate diet of Group A.   Tung oil contains a minimum amount of linolic and linolenic acids, but a high percentage of eleostearic acid, isomeric with linolic acid.

Respiratory Metabolism Determinations

Since the rats of this series were more or less emaciated, the modified formula of Lee (6) $\left(S = 10.76 \times W^{0\cdot61} \times \dfrac{0.310}{N} \right)$ (in which S is surface area measured in sq. cm., W is weight in gm., and N, the degree of emaciation) was used in calculating the surface areas.   This formula includes a correction factor for the degree of emaciation $\left(N = \dfrac{W^{\frac{1}{3}}}{L} \right)$ (in which L is the length in cm. from nose to anus) originally derived by Cowgill and Drabkin (7). Lee, on the basis of his series of emaciated, normal, and obese rats, ascribes the value 0.310 to N for the average rat of normal proportions.   ·

The body weights that were used as a basis for the calculations of surface area were obtained immediately before the beginning of the preliminary fasting period.

The weight of the carbohydrate test meal was based on the calories required by an average normal rat of the same surface area for 12 hours.   As the amount required per sq. cm. of body surface by the average rat for 12 hours on the basis of 800 calories per sq. m. per 24 hours is 0.04 calories, and as 0.009734 gm. of dextrin will furnish this amount of energy, the product of surface area in sq. cm. by 0.009734 ·gives the weight of dextrin required for the test meal (5).   ·

The test meal of dextrin, made into a thin paste with water, was usually eaten readily by the rat within 10 to 15 minutes.   The rat was then placed in the chamber of the closed circuit respiratory calorimeter (5), and the respiratory quotients and metabolic rate

TABLE I

Metabolic Rate and Respiratory Quotients of Rats in Various Stages of the Fat-Deficiency Disease

Run No.	Rat No.	Date and weight	Skin symptoms	E (per cent)		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	9 hrs.	10 hrs.	11 hrs.	12 hrs.	
									Average normal†									
					R.Q.	0.90	0.91	0.89	0.88	0.85	0.82	0.79	0.79	0.77	0.78	0.77	0.77	
					Calories	1072	833	777	728	768	764	765	732	728	793	770	775	
					R.Q.	Basal average 0.733												
					Calories	718												
								Group A (carbohydrate diet)										
1	W 30139	Nov. 14, 116 gm.	+++	30	R.Q.	0.98	0.99	0.85	0.80	0.78	0.77	0.77	0.77	0.77	0.74	0.76	0.76	4½ mos. old
				30	Calories	1077	1244	1248	1006	964	1104	981	1163	968	1084	931	969	
2		Nov. 19, 116 gm.	++		R.Q.	Basal		0.77	0.73	0.73	0.73	0.76		0.74‡				
					Calories			888	862	871	883	949		891				
3	W 30153	Nov. 9, 132 gm.	+++	37	R.Q.	1.05	1.01	1.05	0.88	0.82	0.81	0.79	0.77	0.77	0.77	0.75	0.72	4½ mos. old
					Calories	1291	1477	1115	1358	1248	1199	1090	1234	1184	1068	1250	1063	
4		Nov. 13, 134 gm.	G	34	R.Q.	Basal		0.73	0.74	0.73	0.73	0.74	0.74	0.74				
					Calories			897	902	981	914	868	841	798				
5		Nov. 21, 144 gm.	G	24	R.Q.	0.99	1.05	1.12	0.99	0.88	0.83	0.78	0.78	0.76	0.78	0.78	0.78	Methyl linolate from Nov. 14
					Calories	726	913	910	935	973	886	964	882	895	906	880	895	
6		Nov. 24, 145 gm.	G	23	R.Q.	Basal		0.74	0.72	0.74	0.72	0.74		0.73				" "
					Calories			933	975	861	1012	968		950				

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No.		Animal	E*	Date, weight		Values	Note
7	40	W 30154	++	Nov. 20, 127 gm.	R.Q.	0.97 0.97 1.05 1.03 0.95 0.82 0.81 0.78 0.79 0.77 0.78 0.76	4½ mos. old
					Calories	953 893 931 1007 975 1013 1039 962 1030 1063 999 983	
8	43		+	Nov. 23, 124 gm.	R.Q.	Basal 0.71 0.71 0.71 0.72 0.72 0.74 0.72	
					Calories	953 943 1031 1002 955 **977**	
9	23	W 30155	++	Nov. 12, 125 gm.	R.Q.	0.99 1.01 0.98 1.03 0.87 0.80 0.80 0.79 0.78 0.78 0.76	4½ mos. old
					Calories	991 828 1092 1091 1113 1047 984 1136 1017 998 993 1152	
10	24		++	Nov. 23, 124 gm.	R.Q.	Basal 0.75 0.75 0.72 0.73 0.73	
					Calories	941 888 872 866 879 **889**	
11	27	W 30157	+++	Nov. 10, 128 gm.	R.Q.	1.00 1.04 0.99 1.04 1.06 0.87 0.82 0.81 0.81 0.78 0.77 0.78	4½ mos. old
					Calories	932 910 907 1033 921 1121 910 858 965 1030 921 948	
12	14			Nov. 22, 140 gm.	R.Q.	1.04 1.07 1.04 0.92 0.85 0.82 0.79 0.83 0.75 0.78 0.75 0.76	Methyl linolate
					Calories	1014 910 1094 1182 970 929 1041 860 970 941 907 890	from Nov. 13
13	14			Nov. 25, 139 gm.	R.Q.	Basal 0.76 0.74 0.72 0.71 **0.73**	"
					Calories	908 866 921 893 1028 **923**	
14	31	W 30161	+++	Nov. 15, 142 gm.	R.Q.	0.91 0.93 0.96 0.96 0.97 0.83 0.80 0.79 0.77 0.78 0.79 0.76	4½ mos. old
					Calories	928 983 1009 1012 1054 1114 1176 988 969 1006 962 1020	
15	29		++	Nov. 19, 139 gm.	R.Q.	Basal 0.75 0.76 0.72 0.75 0.73 0.73 **0.74**	
					Calories	895 860 996 966 788 923 **906**	
16	30	W 30167	++	Nov. 11, 131 gm.	R.Q.	1.00 1.02 0.93 0.84 0.79 0.77 0.78 0.77 0.77 0.77	4½ mos. old
					Calories	773 1067 1237 1268 990 990 1201 1036 1140 1227 1027 1134	
17	26		++	Nov. 18, 135 gm.	R.Q.	Basal 0.74 0.77 0.74 0.75 0.75 0.74 0.73 **0.74**	
					Calories	909 813 964 810 960 867 895 571 **895**	

* E measures emaciation.
† The normal rats and those of Group D are male; all others are female.
‡ The averages are given in bold face figures.

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TABLE I—Continued

Run No.	Rat No.	Date and weight	Skin symptoms	E (per cent)*		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	9 hrs.	10 hrs.	11 hrs.	12 hrs.	
									Group A (carbohydrate diet)—Continued									
18	W 30027	Sept. 25, 144 gm.	++	57	R.Q.	1.06	1.06		0.97	0.80	0.78	0.79	0.76	0.77	0.76	0.76	0.79	9 mos. old
					Calories	1059	1139	962	967	1015	874	898	884	911	908	954	795	
19		Oct. 9, 127 gm.	++++	77	R.Q.		1.12	1.12	1.20	1.18	1.12	1.02	0.98	0.92	0.87	0.89	0.91	Test meal, 3 gm. dextrin per 100 gm.
					Calories		684	774	785	890	730	927	942	722	738	655	689	
20		Oct. 17, 113 gm.	++++	90	R.Q.	1.06	1.08	1.08	1.03	0.94	0.89	0.87	0.87	0.88	0.86	0.83	0.86	Died Oct. 18
					Calories	886	758	910	834	926	1027	927	894	946	883	852	809	
21	W 30029	Sept. 22, 115 gm.	++	81	R.Q.	0.97	1.06	1.00	0.99	0.97	0.92	0.82	0.83	0.78				Test meal, sucrose, 400 calories per sq. m. surface
					Calories	767	793	847	855	863	834	910	719	796				
22		Oct. 10, 98 gm.	++++	100	R.Q.		0.80	1.08	1.10	1.08	1.00	0.99	0.94	0.90	0.94		0.92	9 mos. old
					Calories		807	715	742	720	693	682	644	686	672	625	618	
23	W 30028	Sept. 23, 133 gm.	+	61	R.Q.	1.09	1.07	1.06	1.05	1.01	1.02	0.93	0.84	0.80	0.78	0.78	0.78	9 mos. old
					Calories	820	849	732	828	868	799	720	835	754	716	700	76±	
24		Oct. 8, 124 gm.	++	69	R.Q.		1.18	1.10	1.11	1.03	1.02	0.99	0.98	1.01	0.95	0.88	0.87	Test meal, 3 gm. dextrin per 100 gm.
					Calories		562	665	678	682	741	744	715	726	716	661	666	
25		Oct. 16, 113 gm.	++	80	R.Q.	1.11	1.08	1.11	1.08	1.08	1.01	0.88	0.85	0.83	0.81	0.80	0.79	
					Calories	667	697	715	743	700	732	736	706	713	667	726	701	
26		Oct. 22, 131 gm.	+	61	R.Q.	1.15	1.19	1.15	1.05	0.88	0.81	0.82	0.85	0.79	0.78	0.77	0.76	Methyl linolate from Oct. 17
					Calories	919	763	919	915	914	863	850	813	762	724	777	773	

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#	Animal	Date, weight		Day		R.Q. / Calories	Note
27		Nov. 1, 150 gm.	. +	43	R.Q.	1.05 1.07 1.04 0.99 0.97 0.84 0.79 0.78 0.78 0.77 0.77 0.76	Methyl linolate from Oct. 17
					Calories	936 1111 915 848 897 847 803 800 850 828 799 846	

Group B ("treated")

#	Animal	Date, weight		Day		R.Q. / Calories	Note
29	W 29310	Oct. 2, 167 gm.	++	51	R.Q.	0.95 0.96 1.07 0.92 1.01 0.87 0.90 0.85 0.80 0.79 0.78 0.79	11 mos. old
					Calories	940 869 973 892 910 1021 1038 928 919 930 892 910	
31	W 29298	Sept. 27, 168 gm.	G	59	R.Q.	0.89 0.99 1.01 0.93 0.90 0.88 0.83 0.78 0.78 0.77 0.78	13 mos. old
					Calories	1012 898 953 906 833 850 822 835 755 745	
35		Nov. 7, 164 gm.	+	61	R.Q.	1.16 1.05 1.03 0.99 0.86 0.81 0.81 0.78 0.78 0.78	Methyl linolate from Oct. 24
					Calories	1000 1062 1090 1021 892 956 881 908 888 894 1015 1014	
36	W 29309	Oct. 6, 158 gm.	G	66	R.Q.	0.89 1.00 0.93 0.88 0.87 0.90 0.91 0.85 0.79 0.78	13 mos. old
					Calories	618 578 658 706 693 743 749 747 732 736	
41		Nov. 17, 175 gm.	+	53	R.Q.	0.87 1.02 0.98 0.99 1.01 1.01 0.99 0.85 0.83 0.81 0.77	Liver lipoids from Nov. 1
					Calories	734 712 773 769 746 752 752 768 762 752	
43	W 29311	Oct. 20, 148 gm.	++	63	R.Q.	0.99 0.97 1.02 1.04 1.06 0.87 0.81 0.83 0.81 0.79 0.78	13 mos. old
					Calories	949 991 1041 908 992 903 827 900 885 894	
46		Nov. 16, 143 gm.	++++	70	R.Q.	0.84 0.85 0.93 0.94 0.92 0.91 0.87 0.92 0.91 0.88 0.82 0.81	Liver lipoids from Nov. 10
					Calories	755 749 747 788 781 852 828 809 836 818 791 824 814 1014	
47	W 30033	Oct. 5, 125 gm.	+	56	R.Q.	0.94 1.06 1.06 0.97 0.87 0.84 0.82 0.81 0.79 0.79 0.80	1 yr. old
					Calories	572 556 783 786 816 728 686 677 633 709 672	

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TABLE I–*Concluded*

Group D (tung oil with high carbohydrate diet)

Run No.	Rat No.	Date and weight	Skin symptoms	E (per cent)		1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	9 hrs.	10 hrs.	11 hrs.	12 hrs.	
53	W 29308	Oct. 4, 216 gm.	G	47	R.Q.	0.89	0.99	0.96	0.97	1.04	0.87	0.86	0.78	0.81	0.82	0.80		13 mos. old
					Calories	984	860	853	828	798	750	726	677	723	640	654		
57	W 29306	Oct. 3, 186 gm.	G	66	R.Q.	1.04	1.05	1.03	1.00	1.02	0.87	0.85	0.81	0.81	0.84	0.81	0.85	13 mos. old
					Calories	838	832	815	805	831	763	758	784	734	681	739	671	
62	W 29307	Sept. 28, 171 gm.	++	91	R.Q.	0.91	0.93	0.94	0.93	0.88	0.82	0.80	0.80	0.80	0.77			13 mos. old
					Calories	732	728	728	738	720	771	713	787	751	742			
64		Oct. 21, 162 gm.	++	88	R.Q.	1.07	1.12	1.04	1.09	1.09	1.00	0.92	0.86	0.85	0.82	0.81	0.81	Methyl linolate from Oct. 15
					Calories	863	761	774	661	711	722	709	666	633	615	748	735	
65		Oct. 30, 174 gm.	+	79	R.Q.	1.13	1.13	1.06	0.99	0.85	0.84	0.81	0.79	0.79	0.78	0.78	0.77	" "
					Calories	847	813	693	826	770	808	801	766	793	830	825	855	

were then determined at 28° in the same manner as described elsewhere (5).

The time interval is measured from the beginning of the test meal to the average time of the period for which a determination is made. For example, if the interval is 1.0 to 1.9 hours, it is considered as the 1st hour for the purposes of tabulation.

Results

In comparing with each other (Table I) the results of this series of determinations of the metabolic rate and respiratory quotients of rats in various stages of the fat-deficiency disease, weight should be put not only on the severity of the skin symptoms (expressed by +), but also on the percentage of emaciation (E), which is derived as follows: The most severely emaciated rats of our series had an emaciation coefficient (N) of 0.240. Lee's coefficient for the average rat of normal proportions is 0.310. We have used the difference between these coefficients, 0.070, for calculating the percentage of emaciation on the assumption that a rat is 100 per cent emaciated when the emaciation coefficient is 0.240. Therefore, the percentage emaciation (E) used in Table I expresses the degree of emaciation of our animals as the per cent of total emaciation obtained. For example, an animal with an emaciation coefficient of 0.240 is 100 per cent emaciated, and one with a coefficient of 0.310 is 0 per cent emaciated.

The metabolic rate should also be taken into consideration in connection with the rapidity with which the respiratory quotients fall from the initially high values after the carbohydrate meal.

Respiratory quotients above unity are observed in all groups especially with those rats whose emaciation is more rapidly increasing or decreasing as in Runs 5, 12, 18–20, 22, 25–27, 32, 35, 40, 64, and 65. ·   ·

Runs 1 to 17 were made upon rats 4½ months old that had been upon the carbohydrate diet for nearly 4 months, while Runs 18 to 28 were made upon rats 9 months old that had been 8 months upon the same diet. It is to be noticed that the metabolic rate, both assimilatory and basal, is exceptionally high in the fir t series of runs (Runs 1 to 17), compared with the average normal value, and is normal or subnormal in the second series (Runs 18 to 28) just mentioned.

The metabolic rate is also normal or subnormal for the rats of the other groups. The effect of methyl linolate in general is to increase the metabolic rate especially where the emaciation is decreased by the methyl linolate as in Runs 13, 26, 27, 34, 35, and 65.

The rectal temperatures measured with a mercury thermometer, of thirty-one individuals of various groups of rats on the fat-free diet ranged between 37.0 and 38.7°, average 37.7°, while those of normal rats under the same conditions of temperature for 3 days or more varied between 37.5 and 38.0°, average 37.6°. The difference is too small to be considered of significance.

DISCUSSION AND CONCLUSIONS

Formation of Fat from Carbohydrate—The formation of fat from carbohydrate is a normal process of well fed animals whose glycogen stores are practically filled. This conversion of carbohydrate to fat represents a mode of utilization of the excess of ingested carbohydrate in a form that can no longer be retained as such. In the present series of rats, as well as with normal rats with which comparison is made, the carbohydrate stores are presumably no longer full after the preliminary fasting period of 14 or more hours. If normal rats are fed a moderate meal of dextrin under these circumstances, the highest respiratory quotient reached is 1.00, and the average 0.91. Although it is believed that respiratory quotients ranging from 0.90 to 1.00 may represent some degree of fat combustion (8), quotients above 1.00 according to our present knowledge may be taken to definitely indicate the formation of fat from carbohydrate, which proceeds simultaneously with the normal combustion of carbohydrate, protein, and possibly fat. In the present series, judging from the respiratory quotients that are in many cases well above 1.00, we have the conversion of carbohydrate into fat despite the preliminary fasting period. This condition may therefore be considered abnormal.

The diseased animals in their emaciated condition are becoming progressively free from stores of fat in spite of the generous intake of food. As they lose weight on the fat-deficient diet, their loss of fat is excessive. Under the influence of linolic or linolenic acids they gain weight, and the gain in fatty tissue is excessive. It is shown by the respiratory quotients that this fat, at least in part,

is produced from carbohydrate. It is a matter of great interest, however, that linolic or linolenic acids apparently do not result from this conversion, either in the primary stage of formation of fat or in the secondary stage of utilization of this fat, since linolic or linolenic acids from sources outside the body are apparently required to cure the characteristic symptoms of these diseased rats. If they were formed, it would be expected that symptoms cured by linolic and linolenic acids would not develop.

This formation of fat from carbohydrate even after methyl linolate or liver lipoids has been given is noteworthy, since Wesson (4) found under conditions different from those described above a marked although temporary reduction in abnormal fat-forming quotients following the feeding of small amounts of liver lipoids.

Metabolic Rate—The examination of the metabolic rate of these diseased rats comprised the second object of this investigation. This question derived increased interest because of a suggestion put forward by Chidester (9) and by Chidester and Wesson (10) based on the work of others (11–20) that the factor of hyperthyroidism might be found to exist in a condition in which animals were fed a fat-deficient diet for a long period if they received at the same time normal, or even minute amounts of inorganic iodine. The experimental work upon which this suggestion was based supports in a manner the hypothesis that a physiological antagonism between unsaturated acids and the thyroid exists, and that, with the lack of unsaturated acids in the diet, the effect of the thyroid becomes accentuated, or, *vice versa*, with an excess of unsaturated acids in the diet, the effect of the thyroid on the body becomes markedly diminished.

The results of these metabolism experiments are in many respects indicative of the correctness of the surmise mentioned above, although certain features are difficult to reconcile with the conditions that might be expected in hyperthyroidism. In support of the theory of hyperthyroidism as applied to this series of rats may be mentioned the high metabolic rate observed in some cases. This is seen to be present in a pronounced degree in rats just entering the condition recognizable as characteristic of the deficiency disease, and apparently is not connected with the activity of the animal while in the respiratory calorimeter. This, judging from observation, was normal over the 12 hours

duration of the run. Here the assimilatory and basal metabolic rate reached a value 25 per cent or more above the normal value obtained under very similar conditions, although the animals were not receiving what would ordinarily be an excessive amount of iodine.

The difference between the carbohydrate assimilatory rate and the basal rate is also found to be unusually high in the series of animals just entering the diseased condition. If the difference in this case may be said to be due to the specific dynamic action of the carbohydrate, it may, because of its magnitude, be compared with the high specific dynamic action that has been obtained from carbohydrate in cases in which hyperthyroidism was definitely known to exist (18, 19). On the other hand, unlike Miyazaki and Abelin (18) who reduced this high specific dynamic action by fat feeding, we were unable to detect a reduction after feeding unsaturated acids with the diet for a number of days. Furthermore, the high basal metabolic rate is not reduced by methyl linolate or liver lipoids. These facts, together with the fact that other workers have not found the need for highly unsaturated acids to produce their thyroid effects, renders it difficult to accept the suggestion as to the possible participation of a condition of hyperthyroidism in the rats, at least with respect to the unsaturated acids-iodine hypothesis as restated by Chidester (9) and Chidester and Wesson (10). McCarrison (15) and Mellanby and Mellanby (17) found butter most effective, Abelin, Goldener, and Kobori (20) found that oleic acid and mixtures of oleic acid with stearic and palmitic acids were as effective as any other oils, while Burr and Burr (2) found that butter and the saturated acids are ineffective in curing the low fat disease. The development of the symptoms of the fat-free disease by rats on Diet D that includes the highly unsaturated eleostearic acid of tung oil also speaks against the validity of this hypothesis as applied to this disease.

On the other hand, the subnormal metabolic rate found in the case of many of the older animals would indicate a condition of possible hypothyroidism that is consistent with the findings of Gray and Loeb (21) and Gray and Rabinovitch (22) that the feeding of iodides results first of all in a period of hyperactivity of the thyroid followed by a prolonged period of hypoactivity. The effect of excess inorganic iodide feeding on the activity of the thy-

roid of animals is discussed by a number of workers (12, 14, 21–30). It should be pointed out, however, that Gray and Rabinovitch (22) found no marked changes in the thyroid until 10 mg. of KI were fed daily (2000 times our doses), and, except for histological changes in the thyroid, the animals were perfectly normal. Their experiments were done with guinea pigs and extended over a period of 108 days. This work would indicate that none of the symptoms of the fat-deficiency disease can be produced by KI feeding.

The abnormal formation of fat from carbohydrate (respiratory quotients above unity) has been found by Miyazaki and Abelin (18) in the case of rats in a condition of hyperthyroidism. Whether fat is formed from carbohydrate also in the case of emaciated animals with a known subnormal thyroid activity has not been determined, so far as we are aware.

Emaciation (31–34), cessation of growth (35), hemorrhage (33, 34), sterility (36), excessive consumption of water, high food intake (31, 33, 37), and the lack of apparent nervousness or tendency to excessive activity (38), are features of the condition of these rats that are compatible with what would be expected in a condition of hyperthyroidism. A difference of opinion is found in the literature as to body temperature in hyperthyroidism (31, 34, 39, 40).

The contradictory evidence as to the thyroid phase, if any, of the fat-deficiency disease awaits elucidation from other angles. This work is now being undertaken as a part of the larger problem mentioned in the introductory paragraphs.

SUMMARY

1. The metabolic rate and respiratory quotients following a carbohydrate test meal have been determined in the case of rats maintained for some time on a fat-deficient diet, and are compared with those obtained on normal rats and under approximately the same conditions.

2. The respiratory quotients in the 1st hours following the carbohydrate feeding are in many cases well above unity, definitely indicating the formation of fat from carbohydrate by these rats in various stages of the fat-deficiency disease.

3. The fact that no relief is obtained from the symptoms of the fat-deficiency disease by the fat thus formed from carbohydrate

indicates that the curative linolic and linolenic acids are not formed by the rat from the carbohydrate or from the fat.

4. The basal and assimilatory metabolic rate in the case of rats showing the early symptoms of the fat-deficiency disease was well above the normal value, while the metabolic rate in the later stages of the disease was normal or subnormal.

5. The possible relationship of thyroid activity to several phases of the fat-deficiency disease is discussed.

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THE TETRACOSANIC ACID OF PEANUT OIL

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Lignoceric acid was first recognized in 1888 by Hell and Hermanns (1). It owes its name to the fact that it was found in beech wood tar. In 1896 Kreiling (2) isolated it from peanut oil which has become the usual source and of which it is the characteristic acid. Since its discovery it has served as an important reference compound for the higher fatty acids. Many investigators have prepared it with a melting point of 81° and of the higher acids it has been the one most definitely characterized. Cerotic, montanic, and other higher acids have shown considerable variation in melting point and even in molecular weight.

Lignoceric was originally thought to be a normal acid but in 1913 Meyer, Brod, and Soyka (3) showed that the docosanic acid to which it gave rise on degradation was not identical with behenic acid and it has been considered, as a result of that work, to have a branched chain of carbon atoms.

Brigl and Fuchs (4) in 1922 fractionated old beech wood tar and obtained a normal acid melting at 85° and a second tetracosanic acid melting at 74°. They concluded that lignoceric acid melting at 81° was an impure normal acid. Their fractionation was repeated exactly by Levene, Taylor, and Haller (5) on the lignoceric acid of peanut oil. The latter were unable to alter the melting point and the status of the acid from that source therefore remained unchanged.

The question of the structure of lignoceric acid is of immediate importance in the elucidation of the structures of the acids of the cerebrosides. Klenk (6) has recently prepared from the latter a tricosanic acid and Taylor and Levene (7) have related it to the lignoceric series. Furthermore Klenk (8) has obtained lignoceric acid by the reduction of cerebronic acid.

In the experiments to be described (9), the higher acids of peanut oil were fractionated by means of both distillation and crystallization. From the higher fractions that have been examined, there has been isolated only one pure individual acid, *n*-tetracosanic. It melted at 84–85° and, mixed with an equal quantity of the synthetic *n*-tetracosanic acid of Levene and Taylor (10), melted at 84.4–85.4°. The synthetic acid melted at 84.6–85.6°.

Certain fractions have tended toward a higher molecular weight (383) and undoubtedly they were made up, at least in part, of the acids described by Holde and Godbole (11) and by Holde, Bleyberg, and Rabinowitch (12). In all other fractions the purifications have taken the direction of *n*-tetracosanic acid.

With continued fractionation the elevation of the melting point has in no case stopped at 81°. There is therefore no indication of the presence of the acid known as lignoceric and it would appear that that substance is a mixture of several acids, that is very resistant to separation by crystallization. There is already other evidence pointing in the same direction. Among the fractions obtained by Taylor and Levene (7) from the oxidation product of cerebronic acid was one that approximated a tricosanic acid in composition. When that acid, melting at 76.5–77.5°, was mixed in about equal proportion with the tricosanic acid, melting at 76–77°, obtained by the removal of 1 carbon atom from the lignoceric acid of peanut oil, the melting point of the mixture was not depressed. Moreover, the acid derived from the cerebroside was shown to be made up of more than one individual acid. Either the melting point of the mixture, in determining the identity of two specimens, is without value in this instance or both of the components must be of similar composition.

The melting points of a series of acids homologous with lignoceric offer a further interesting observation. As pointed out by Taylor and Levene (13), those of both the even and the odd numbered members fall on a smooth curve, whereas two separate curves represent the melting points of the odd and even series of normal acids. The relationship existing in the lignoceric series could be explained as the result of branched chains in individual acids or as due to the presence of several acids in each member. The latter state is represented in the paraffins obtained by fractionation of petroleum.

Of course the only absolute proof that lignoceric is made up of several individual acids is to be found in the preparation of the substance from peanut oil and fractionation leading to the isolation or at least the partial separation of the components. That work is now in progress.

Methods

The melting points recorded were taken with a short stem, standardized thermometer and are corrected. A few mg. of the material to be tested were melted, over a small flame, on a watchglass and were allowed to cool in an evacuated desiccator. Solvents were completely removed by this treatment but, as a further precaution, it was repeated twice before the melting point was determined. The heating was regulated to raise the temperature 1° in 7 to 8 seconds. The bath was well stirred. The solidification points were obtained by allowing the bath to cool with stirring until the material in the tube was solid. The recorded points indicate the temperatures at which the first crystals formed and at which solidification was apparently complete. The equivalent weights, which are reported as molecular weights, were obtained as described by Taylor and Levene (13) and are 2 to 3 units higher than the actual values. All distillations were conducted at a pressure of 0.07 to 0.10 mm. of mercury, the temperature being so adjusted as to collect the distillate at the rate of 1 drop in 6 to 8 seconds. The metal bath temperature was usually about 60° above that of the vapor in the neck of the bulb.

Fractionation—Two samples of peanut oil served for the preparation of the fatty acids used in these fractionations. The water-insoluble acids from the first lot were supplied to us by Colgate and Company. The second lot was saponified in this laboratory. The fat (500 gm.) was treated with sodium hydroxide (290 gm. of 50 per cent solution) and 95 per cent alcohol (200 ml.) and boiled for a half hour. Steam was then passed through until the alcohol was almost completely removed and the mixture was beginning to foam badly. The soap was dissolved by the addition of water and by heating, and then slightly more than an equivalent of diluted sulfuric acid was added. Steam was again passed through until the upper layer was liquid, after which two lots of oil were united

and again steamed with acidulated water until the upper layer was transparent. Steaming with water several times—until the washings were no longer acid to Congo red—removed the excess of sulfuric acid. In this way 3208 gm. of insoluble fatty acids were obtained from 3370 gm. of oil.

The description of the separation of the higher acids from the above mixture applies particularly to the treatment of the second lot. The first lot was worked up in a similar manner. The method used has been briefly mentioned before by Levene, Taylor, and Haller (5).

The liquid acids were cooled overnight in 500 ml. centrifuge bottles to 22° and centrifuged at about 1000 r.p.m. until the volume of the separated solid was nearly constant. 30 minutes were usually sufficient. The sediment in each bottle was then broken up in alcohol (100 ml.) and again centrifuged, after which the moist solid weighed 125 to 130 gm. The acids in two bottles were united, treated with alcohol (125 ml.), and again centrifuged. The moist sediment now weighed about 180 gm. After three similar washings with alcohol the total yield had been reduced to 354 gm. or 11 per cent of the insoluble acids.

The yield of solid acids could have been decreased or increased somewhat by cooling to a higher or lower temperature before centrifuging. These were cooled to room temperature which was usually very close to 22°. The temperature of the supernatant oil after the sediment had been packed was 23–24°.

This method of recovering the higher acids from peanut oil presents considerable advantage over the other procedures that have been described. Both labor and solvent are conserved. The alcohol required for the separation to this point was about 3600 ml. Of this 1500 ml. were used in the saponification and the remainder for washing the solid acids in the centrifuge.

The acids were then crystallized from 95 per cent alcohol at 24° after which the melting point was 72.5–73.5° with resolidification at 71.5–71°. The material was colorless and beautifully crystalline. Two further crystallizations brought about no change in the melting point. The yield was 78.5 gm. or 2.45 per cent of the water-insoluble acids. The acid did not absorb iodine. The molecular weight was 351.

0.5001 gm. substance required 7.12 ml. 0.2 N NaOH.

The acid (73.5 gm.) was dissolved in 99.5 per cent alcohol (1000 ml.) to which sulfuric acid (10 ml.) had been added and the solution was boiled overnight. The ester was crystallized at 0° and recrystallized from 95 per cent alcohol (800 ml.) at 0°. It melted at 50.5–51.5° and resolidified at 50–49.5°. The yield was 76.6 gm. or 96.5 per cent of the theoretical. The final mother liquor was neutral to litmus.

In order to diminish the heating to which the ester was subjected, it was divided into two equal parts and these were distilled separately. The corresponding fractions were then united.

TABLE I

First Fractional Distillation of Esters of the Solid Acids

Fraction	B.p.	Yield		Total yield
		1st distillation	2nd distillation	
	°C.	gm.	gm.	gm.
Distillate I*	204–205	5.8	4.5	10.3
" II	204–205	4.4	7.5	11.9
" III	206–207	4.1	5.6	9.7
" IV	206–207	5.6	4.2	9.8
Residue I†		8.8	8.6	17.4
" II		6.0	4.2	10.2
" III		3.7	3.6	7.3

* Distillate of greatest volatility.
† Residue of least volatility.

The plan of fractionation was that adopted by Taylor and Levene (7). A small fraction of greatest volatility was collected. Then a large middle fraction was distilled, leaving a small residue of least volatile material. The large middle fraction was redistilled in the same manner and the process repeated until the whole specimen had been distributed in fractions of the desired size. In Table I are to be found the data for the fractions obtained by distilling these two lots of ester.

With the distillates of Table I we have no further concern; their treatment will be reported at another time. The residues were distilled into three successive fractions each, two distillates and a residue. The esters were then saponified by heating on the water bath with alcoholic sodium hydroxide; the alcohol was

removed and the acids were liberated by heating the soaps with dilute hydrochloric acid on the water bath. The acids after crystallization from acetone at 0° were all beautifully crystalline. In Table II are given the physical constants of the acids as well as the boiling points of the esters.

It may be noted that the esters from which these acids were derived contained very little free acid. If free acid had been present, the molecular weights of the final residues, and especially of Residue I C, would have been decreased since the average molecular weight of the whole specimen was considerably lower.

TABLE II

Redistillation of Residues of Table I

Fraction*	B.p. of ester	Yield of acid	Mol. wt. of acid	M.p. of acid	Solidification
	°C.	gm.		°C.	°C.
Residue I A	214–216	4.0	358	74.5–75.5	73.5–73
" I B....................	216–218	4.9	361	76–77	75–74.5
" I C....................	Residue	7.0	375	76.5–77.5	76–75.5
" II A....................	216–219	3.8	355	74.5–75.5	73.5–73
" II B....................	218–221	3.1	361	76.5–77.5	75.5–75
" II C....................	Residue	1.2	370	77.5–78.5	76.5–76
" III A	210–212	2.0	351	74.5–75.5	72.5
" III B..................	212–215	2.4	356	74.5–75.5	73.5–73
" III C..................	Residue	2.4	368	78.5–79.5	76.5–76

* Residue I weighed 17.4 gm.; Residue II, 10.2 gm.; Residue III, 7.3 gm.

The acids of Table II were now grouped according to molecular weight and repeatedly crystallized from ether. Residues I B and II B were combined and twice crystallized from ether (100 ml.) at 25°. Residue III C was then added and the treatment repeated at 0°. The top fraction then melted at 80.8–81.8°, solidified at 79–78°, and had a molecular weight of 375.

0.4999 gm. substance required 6.67 ml. 0.2 N NaOH.

It was crystallized four times from ether (100 ml.) at 3–5° and then melted at 81.6–82.6° and resolidified sharply at 78.5°. The yield from 10.4 gm. was 3.3 gm. The molecular weight was 374.

0.5001 gm. substance required 6.69 ml. 0.2 N NaOH.

Residues I A, II A, III A, and III B were combined (12.2 gm.) and thrice crystallized from ether (150 ml.), twice at 25° and then once at 3°. The product melted at 81.8–82.8° and had a molecular weight of 372.

0.4999 gm. substance required 6.72 ml. 0.2 N NaOH.

Four more crystallizations from ether (100 ml.) at 3–5° brought the yield to 1.1 gm. and raised the melting point to 83.2–84.2° with resolidification at 81–80.0°. The molecular weight was 373.

0.5006 gm. substance required 6.72 ml. 0.2 N NaOH.

From Residues I C and II C in the same manner, a top fraction of 4.9 gm. was obtained by two crystallizations from ether (100 ml.). It melted at 78.8–79.8° and resolidified at 77–76.5°. The molecular weight was 383.

0.5014 gm. substance required 6.55 ml. 0.2 N NaOH.

Four further crystallizations at 3–5° raised the melting point to 79.2–80.2° with resolidification at 77.5–77°. The molecular weight was still 383.

0.4997 gm. substance required 6.52 ml. 0.2 N NaOH.

Unlike the other fractions this material tended toward a higher homologue than tetracosanic acid (molecular weight 368). It is included to indicate that no part of the fractionated material would give rise to lignoceric acid melting at 80–81° and will not be dealt with further here.

So far the fractionations described have applied only to the acids from the second lot of peanut oil. The total yield of material melting at 82° or higher and with molecular weight approximating that of tetracosanic acid was about 5 gm. This included a small amount obtained by working up the higher mother liquors.

The acids from the first lot of oil were treated in a similar manner. The difference was mainly in that this earlier fractionation was not so well ordered and direct. The products, however, were much like those described above. The best fraction (2.4 gm.) melted at 84–85°, resolidified at 82.5–81°, and had a molecular weight of 372.

0.5006 gm. substance required 6.73 ml. 0.2 N NaOH.

As in the case of the other lot of peanut oil, some acid higher than tetracosanic was obtained.

The total yield from this lot of acid, melting at 82° or higher and of molecular weight of about 370, was 8.5 gm. All of the solid acids were beautifully crystalline.

The products from the two lots of oil (5 and 8.5 gm.) were united, esterified in the usual manner, and fractionally distilled. As in the earlier fractionations, small distillates and residues were successively removed and the middle fractions redistilled until all of the ester was distributed in fractions of the desired size. Seven fractions were obtained. Their boiling points and

TABLE III

Fractional Distillation of the Ester from 13.5 Gm. of Nearly Pure Tetracosanic Acid

Fraction	B.p. of ester	Yield of acid	Mol. wt. of acid	M.p. of acid	Solidification
	°C.	gm.		°C.	°C.
Distillate A*	204–206.5	1.2	367	82.5–83.5	80.8–80.4
" C	206–207	1.3	367	83–84	81.5–81
" E	204	2.0	368	83.5–84.5	81–80.5
" G	203–204	2.8	368	83.4–84.4	81.5–81
Residue B†		2.0	370	80.5–81.5	79–78.5
" D		0.9	373	81–82	79.5–79
" H		3.1	371	82.5–83.5	81–80.5

* Most volatile distillate.
† Least volatile residue.

the physical constants of the acids to which they gave rise are shown in Table III.

The tetracosanic acid was contaminated by acids of both higher and lower molecular weight. Further purification was effected by crystallization from ether. Distillates A and C were united (2.5 gm.) and twice crystallized from ether (75 ml.) at 7°. The acid (2.2 gm.) then melted at 83.5–84.5° and resolidified at 81.5–81°. Three crystallizations of Residue H from ether (75 ml.) at 7° brought that fraction to exactly the same condition. The yield was 2.5 gm. Residues B and D, combined, were but slowly changed by this treatment and were not treated further.

Distillates A, C, E, G, and Residue H (9.5 gm. in all) were

united and crystallized repeatedly from ether (200 ml.) at 5–6°. The melting point rose slowly to 84–85° with resolidification at 82.2–82°. The yield was 8.0 gm. after four crystallizations. Three further crystallizations reduced the yield to 6.9 gm. without altering the melting and solidification points.

0.4973 gm. substance: 6.73 ml. 0.2 N NaOH.
0.1001 "   "   : 0.2865 gm. CO_2 and 0.1170 gm. H_2O.
    Calculated for $C_{24}H_{48}O_2$. C 78.26, H 13.04, mol. wt. 368
    Found.   " 78.05, " 13.08, "   " 369.5

The acid was mixed in approximately equal proportions with a specimen of synthetic n-tetracosanic acid, for which I wish to express my thanks to Dr. P. A. Levene. The melting point of this mixture was determined simultaneously with those of the components, with the results shown in Table IV.

TABLE IV

Melting Points of n-Tetracosanic Acid, of Acid from Peanut Oil, and of Their Mixture

	M.p.	Solidification
	°C.	°C.
Synthetic n-tetracosanic acid..................	84.6–85.6	81.5
Tetracosanic acid from peanut oil.............	84–85	82
1:1 mixture (approximate)....................	84.4–85.4	82

The methyl ester was prepared by boiling the solution of the acid in absolute methyl alcohol with sulfuric acid. It was crystallized from methyl alcohol and melted at 58.5–59.5°. The ester prepared from the synthetic acid by Levene and Taylor (10) melted at 59–60°.

0.1001 gm. substance: 0.2888 gm. CO_2 and 0.1171 gm. H_2O.
    Calculated for $C_{25}H_{50}O_2$. C 78.54, H 13.09
    Found.   " 78.67, " 13.09

The ethyl ester, prepared in a similar manner, melted at 55–56°. Levene and Taylor (10) found a melting point of 55.5–56.5° for the ester of the synthetic acid.

0.1000 gm. substance: 0.2885 gm. CO_2 and 0.1167 gm. H_2O.
    Calculated for $C_{26}H_{52}O_2$. C 78.79, H 13.13
    Found.   " 78.67, " 13.06

SUMMARY

1. The higher saturated acids of peanut oil have been systematically fractionated by both crystallization and distillation.

2. *n*-Tetracosanic acid has been isolated.

3. No evidence of the presence of lignoceric acid, melting at 81°, has been found.

4. The question of the individuality of lignoceric acid has been discussed.

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THE FREEZING POINT DETERMINATION OF PHYSIO-LOGICAL SOLUTIONS

THE USUAL ERRORS AND THEIR ELIMINATION

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This paper outlines a method for making freezing point determinations of small amounts of physiological solutions (1 cc.) without unusual complication of apparatus and technique. With the use of this procedure supercooling is limited to a few hundredths of a degree and the temperature of the cooling bath is kept within a few tenths of a degree of that of the freezing point. The desired degree of supercooling is brought about without excess supercooling and without causing serious dilution, by the use of small metal rings cooled in CO_2 snow, so that a considerable number (twenty to thirty) of repeated determinations can be made with the same 1 cc. sample of a solution.

The most refined technique which has been applied for determining the freezing point of salt solutions is that in which a solution is brought into equilibrium with comparatively large amounts of ice crystals, after which the equilibrium temperature is noted and a sample is withdrawn to determine the solution concentration at that particular point (1). It is obvious without going into detail that such a procedure is not applicable to solutions like those of blood sera, especially when small amounts only are available and changes in concentration are undesirable.

Fromm and Leipert (2) have recently used a freezing point method which can be used with as little as 3 cc. of blood serum. They allowed a difference of $1.0°$ between the temperature of the cooling bath and that of the freezing point of the serum and allowed the experimental solution to supercool $0.5°$ to $0.6°$. As will be shown later, this temperature difference and the amount of supercooling are too great to obtain the most accurate results.

The errors of excessive supercooling and those of keeping the temperature of the cooling bath too low are well known. It is obvious that these errors become greater when the amount of experimental solution is greatly reduced. When smaller amounts of solution are used the heat capacity of the *apparatus* becomes relatively large and cooling of the experimental solution goes on at an increased rate. Supercooling the experimental solution 0.8° alone should produce an error of 1 per cent because of the increased solution concentration due to the formation of ice crystals. This error would be increased considerably when the heat capacity of the apparatus is relatively large. Keeping the temperature of the cooling bath too low accelerates the formation of ice crystals, causing concentration of the experimental solution, and consequent excessive lowering of the freezing point. This makes it difficult to obtain reproducible results. When the difference between the temperature of the cooling bath and that of the freezing point of the experimental solution is greater than 1.0°, these errors are pronounced even though as much as 15 cc. of the experimental solution are used and supercooling is limited to a few hundredths of a degree. It is usually thought that these errors are eliminated because they apply equally to determining the freezing point of solutions and of pure solvents. In the latter case, however, changes due to changes in solution concentration do not take place. The generally accepted statement, that good results can be obtained without special precautions even when the convergence temperature is several degrees lower and when supercooling is not more than 1.0° (3), should be applied only to the case of pure solvents. The correction formula which has been applied for excessive supercooling cannot be used when the amount of heat abstracted by the *apparatus* is relatively large as must be the case when relatively small amounts of experimental solutions are used.

In the present experiments it was found desirable to maintain a difference in temperature of 0.4° between the cooling bath and the freezing point of the experimental solution and to limit supercooling to a few hundredths of a degree.

<div align="center">EXPERIMENTAL</div>

The cooling bath, Fig. 1, consists of a wide mouth thermos flask (27 cm. deep × 6 cm. inside diameter) containing a 25 per cent

aqueous solution of alcohol. It is fitted with a cork stopper bored at b to hold a thermometer, B, for measuring the temperature of the bath; at c to admit the stem of a loop stirrer, C; and at d to hold a large test-tube, D (28 cm. deep $\times$ 2.7 cm. inside diameter). A fourth hole admits small pieces of CO_2 snow (dry ice) for regulating the bath temperature. The chamber E, which is open at the constricted end, has the function of making it possible to accelerate cooling of the experimental solution in the chamber F, so as to bring its temperature near the freezing point without excessive delay. It is sealed air-tight to the tube F by means of a rubber connection at g. Without this device the time required to bring the temperature of the experimental solution near the freezing point would be excessively long when the difference in temperature between the cooling bath and the freezing point of the experimental solution is kept at about $0.4°$. The tube D contains 10 to 15 cc. of a 25 per cent alcohol-water solution. By applying suction at the side neck e, this cooled solution is drawn into E, bringing it into contact with the tube F, thus rapidly lowering the temperature of the experimental solution. When the alcohol-water is again allowed to recede the tube E provides a double air space between the cooling bath and the experimental solution.

The chamber F containing the experimental solution is of Pyrex glass. It is 27 cm. deep and the upper part has an inside diameter of 1.6 cm. The lower constricted part at f is 5 cm. long and has an inside diameter of 1 cm. The bulb and lower stem of the thermometer H have a diameter of 0.9 cm. The inside diameter of the container at f was chosen so that stirring could be efficiently carried on by moving the freezing point thermometer up and down a distance of about 1 cm. This also provided for the tapping of the thermometer which is required for a proper reading. The freezing point thermometer (Thomas No. 7272) was graduated in $0.01°$ divisions within a range of $+ 1.0°$ to $-5.0°$. The bath temperature thermometer was graduated in $0.1°$ divisions.

The motion of the thermometer for stirring the experimental solution was controlled by the hand of the operator. The cooling bath was stirred mechanically and continuously with the air motor of a windshield wiper. Supercooling was controlled by the use of small nichrome (or platinum) wire rings (made by winding a
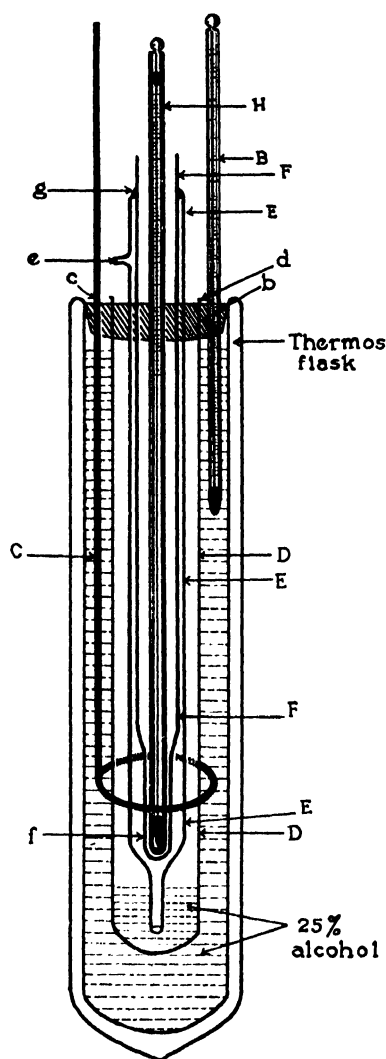
Fig. 1. Cooling bath consisting of a wide mouth thermos flask (containing alcohol) fitted with a cork stopper bored at b to hold thermometer B, at c to admit the loop stirrer C, and at d to hold test-tube D. E, a chamber open at the constricted end is sealed air-tight to tube F by rubber connection g. The thermometer H is set in the constricted part f of chamber F containing the experimental solution which is cooled by contact with cooled solution of E brought in contact with F by applying suction at side neck e.

piece of wire 0.65 mm. in diameter about a 10 gauge wire and cutting single loops as needed) cooled in dry ice. After one preliminary trial to note the approximate freezing temperature of the experimental solution, it was possible to induce freezing of this solution without supercooling beyond a few hundredths of a degree. Before dropping any of the rings into the solution they were exposed to the moisture of the air to allow the formation of a layer of frost. The effect which this amount of moisture might have in repeated determinations with the same 1 cc. sample of experimental solution was determined by weighing ten of these

TABLE I

Data Obtained in Determining Freezing Point of 0.2 M Sodium Chloride Solutions under Various Conditions

Amount of solution	Temperature of cooling bath	Difference in temperature of cooling bath and freezing point	Amount of supercooling	Freezing point observed	Freezing point from International Critical Tables	Freezing point according to Loomis (4)
cc.	°C.	°C.	°C.	°C.	°C.	°C.
1	−2.8	2.1	0.99	−0.740	−0.685	−0.68779
15	−2.8	2.1	0.99	−0.710		
1	−2.8	2.1	0.05	−0.735		
15	−2.8	2.1	0.02	−0.705		
1	−2.3	1.6	1.02	−0.725		
1	−2.3	1.6	0.04	−0.715		
15	−1.9	1.2	0.02	−0.700		
1	−1.6	0.9	0.85	−0.725		
1	−1.6	0.9	0.01	−0.705		
1	−1.1	0.4	0.29	−0.708		
1	−1.1	0.4	0.02	−0.690		
15	−1.1	0.4	0.02	−0.690		

rings before and after they were frosted. The gain in weight due to frost formation on one ring was 0.0001 gm. It is evident that the use of a considerable number of rings would be required to produce an appreciable dilution.

The zero point of the thermometer was determined by the temperature of pure water in equilibrium with ice crystals.

Table I contains data, obtained in determining the freezing point of 0.2 M sodium chloride solutions, under various conditions.

These data were obtained after a large number of preliminary experiments with salt solutions of various concentrations and with

sera.   The error due to a large difference between the temperature of the cooling bath and the freezing point of the experimental solution is more pronounced than the error of excessive supercooling. Both errors are especially great with small amounts of the experimental solution.   This illustrates that relatively large amounts of heat are abstracted by the apparatus under these conditions. As the difference between the temperature of the cooling bath and the freezing point of the experimental solution becomes less, the error of using small amounts of the experimental solution gradually disappears while the error of excessive supercooling persists. It is to be noted that even at this point supercooling but a few tenths of a degree has a marked effect on the freezing point of the experimental solution when small amounts are used.   When supercooling is limited to a few hundredths of a degree and the difference between the temperature of the cooling bath and the freezing point of the experimental solution is 0.4°, the value of the freezing point of even small amounts of the experimental solution approaches that obtained with larger volumes by the use of precision methods.

SUMMARY

The method for making freezing point determinations has been modified so that supercooling is easily controlled and can be limited to a few hundredths of a degree by the use of small rings (of platinum or nichrome wire), supercooled in solid CO_2 and covered with frost.

The usual type of apparatus has been modified, making it possible to make determinations rapidly when the cooling bath temperature is but a few tenths of a degree lower than the freezing point.   The error due to the heat capacity of the apparatus is thus largely eliminated.

The modified method can be used with 1 cc. of solution and is especially adaptable for use with physiological solutions where small amounts are available and dilution is undesirable.   A large number of repeated determinations can be made with the same sample without danger of dilution.

Experimental data are given to show the errors introduced when the temperature of the cooling bath and the amount of supercooling is that usually recommended and employed.

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STUDIES ON THE HEMICELLULOSES

I. THE EVOLUTION OF CARBON DIOXIDE BY PLANT MATERIALS AND SOME HEMICELLULOSES UNDER THE ACTION OF BOILING TWELVE PER CENT HYDROCHLORIC ACID

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INTRODUCTION

The term hemicellulose, introduced by Schulze (1) has in the past been used to designate any polysaccharide that is removed from plant materials by dilute alkali but not by water, and is readily hydrolyzed by hot dilute mineral acids at atmospheric pressure.

Investigators of these substances have been handicapped by the difficulty of preparing them in pure condition (2). As a result, some important constituents have often been overlooked. How-ever, our knowledge of the hemicelluloses has now become sufficient to enable us to classify some of them chemically (3) as well as to draw plausible conclusions as to the possible mechanism of their union in plant materials (4).

The fact that hemicelluloses are dissolved out of plant materials by alkali might suggest that they either contain an acid group and are combined by this to some constituent, possibly the cellulose, of the plant materials, or that they are held in the plant by a

glucosidic union.   In either case alkali might break the union and set the hemicellulose free.   In fact, both kinds of union are probably present in plant materials and thus give rise respectively to (a) acid hemicelluloses and (b) polysaccharide hemicelluloses. The former class is probably similar in structure to the plant gums while the latter class is similar to certain of the true hexosans and pentosans.   Possibly some forms of gummosis in plants are merely hydrolysis of the woody material and liberation of the hemicellulose with little change in composition.

Many investigations reported during the past 20 years dealing with the pectins,[1] the plant gums (5), hemicelluloses (6), certain specific polysaccharides (7), and some plant mucilages (8) have shown the wide distribution (9) and great importance of the uronic acids in plant materials.   Although all acid hemicelluloses so far studied have been found to contain a uronic acid, the fact that agar (10) contains an acid not belonging to the uronic acid group would suggest that not all acid hemicelluloses contain uronic acids.   Many hemicelluloses have been described as pure polysaccharides and undoubtedly many of them really yield only sugars on hydrolysis.   However the work of Butler and Cretcher (11) on Salkowski's araban indicates the need of reinvestigating some of the hemicelluloses which have been reported as yielding only sugars on hydrolysis.

Object of Investigation and Discussion of Methods Used

The investigation was undertaken to determine how widely the uronic acids are distributed in vegetable materials that yield hemicelluloses as well as in the hemicelluloses themselves.

Two methods were used in testing for the uronic acids.   The first of these is quantitative and consists in heating the material with 12 per cent hydrochloric acid according to the method of Lefèvre and Tollens (12) and determining the per cent carbon dioxide evolved.   The apparatus used was similar to that described by Dickson, Otterson, and Link (13), but the heating was limited to 4 hours.   The second method is that of Tollens (14) and depends on the use of naphthoresorcinol.

[1] An extended bibliography of the work on pectins is given in the monograph by Branfoot (Branfoot, M. H., A critical and historical study of the pectic substances of plants, His Majesty's Stationery Office, London (1929)).

The first of the above methods is very general in its application. Uronic acids yield 22.5 per cent of carbon dioxide. Hence any plant material that contains a uronic acid should yield appreciable quantities of carbon dioxide under the conditions of the determinations. However, the method has the defect that carbonates also yield large amounts of carbon dioxide, while some other acids, and even sugars themselves, yield small amounts of the gas.[2]

Since most carbonates are immediately decomposed by 12 per cent hydrochloric acid while uronic acids are only decomposed by boiling the solution strongly, the error due to the presence of carbonates can be easily rectified by adding the 12 per cent acid to the material and warming to approximately 70° for a short time while drawing a stream of air through the solution. When this determination was made on cottonseed hull bran, corn-cobs, white birch sawdust, and white spruce sawdust the results indicated that no more than traces of carbonates were present.

Link[2] has determined the per cent carbon dioxide evolved by a number of pure substances when heated to 135° with 12 per cent hydrochloric acid for 5 hours. He found that oxalic acid gave 0.8 per cent carbon dioxide, four amino acids gave only traces of the gas, while the different sugars gave amounts varying approximately within the limits of 0.2 to 0.5 per cent. The amount of carbon dioxide evolved by sugars depends on the duration of heating and is probably due to oxidation by the air that is drawn through.

The per cent of carbon dioxide evolved by a uronic acid is so much greater than that evolved by any other organic substance likely to be present in plant materials that for most purposes the evolution of large quantities of carbon dioxide under the conditions described may be considered as very strong evidence for the presence of a uronic acid. Evidently the presence of large amounts of either oxalic acid or free sugar renders the test less conclusive. When the amount of carbon dioxide is 0.5 per cent or above and the heating is not continued for more than 4 hours the test is fairly conclusive. For amounts of carbon dioxide less than approximately 0.3 per cent and especially where the hydrolysis leads to the liberation of large percentages of sugars the test is not conclusive but indicates the absence of uronic acids.

[2] These data are to be published by K. P. Link within a short time.

The naphthoresorcinol test (14) for uronic acids was negative when applied to hemicelluloses unless considerable care was used in making it. If small amounts of the hemicelluloses were used the test was positive in all cases where a uronic acid was found to be present. The test is much more conclusive when applied to the salts formed by hydrolysis of the hemicellulose and in general this procedure was followed.

Preparation of Materials

The following plant materials were used in the investigation: cottonseed hull bran, corn-cobs, vegetable ivorynut waste, sawdust from white birch (*Betula sp.*), white spruce (*Picea canadensis*), hickory (*Hicoria ovata*), catalpa (*Catalpa speciosa*), and Western larch (*Larix occidentalis*). The cottonseed hull bran was coarsely ground; the vegetable ivorynut waste was in the form of fine shavings; the corn-cobs were powdered but not sieved; the sawdusts were powders passing an 80 mesh sieve.

In all cases the percentage moisture was determined on a small sample of the material by heating to 108° in an oven. The materials listed above were treated as described below and carbon dioxide determinations made on the untreated materials as well as on the modified products. (1) They were mixed with 15 times their weight of 0.5 per cent ammonium oxalate solution and heated in the boiling water bath for 2 hours. They were then filtered and the residues extracted twice more in the same way with ammonium oxalate solution. Finally the residue was washed thoroughly with hot water. This process should remove the pectins and water-soluble material. (2) Some of the material previously extracted with 0.5 per cent ammonium oxalate solution as in (1) above was mixed with 50 times its weight of 1 per cent sodium hydroxide solution and heated for 1 hour in the boiling water bath, filtered, and the residue thoroughly washed with distilled water, cold 10 per cent acetic acid, and hot water again (15). (3) Some of the material previously extracted with 0.5 per cent ammonium oxalate as in (1) above was mixed with 8 times its weight of cold 5 per cent sodium hydroxide and let stand at room temperature for 48 hours. It was then filtered and washed with water, 10 per cent acetic acid, and water again. (4) Some of the untreated material was mixed with 12 times its weight of 7

per cent sodium hydroxide solution and heated in the boiling water bath for 3 hours. It was then filtered and washed with water, 10 per cent acetic acid, and water again.

Hemicelluloses were prepared from certain of the materials as described below and carbon dioxide determinations made on these (Table I).

1. The untreated material was mixed with 8 times its weight of 5 per cent sodium hydroxide solution and allowed to stand at room temperature for 48 hours. The solution was filtered through several thicknesses of cloth, the filtrate acidified by dilute hydrochloric acid, and approximately 3 times its volume of 95 per cent ethanol added. Finally it was filtered on a Buchner funnel and washed repeatedly with 95 per cent ethanol and dried on a porous plate. This hemicellulose would contain any pectin present in the material.

2. The untreated material was extracted by ammonium oxalate solution as already described, in order to remove pectin. Then the hemicellulose was extracted by cold 5 per cent sodium hydroxide solution as described under (1) above.

3. The untreated material was first extracted three times with boiling 0.5 per cent ammonium oxalate, then one time either with cold 5 per cent sodium hydroxide for 48 hours or with boiling 1 per cent sodium hydroxide for 3 hours. This should remove all the pectin and the easily soluble hemicellulose. The residue was next extracted by 7 per cent sodium hydroxide solution in the boiling water bath for 3 hours, the solution filtered through several thicknesses of cloth, and the hemicellulose isolated as described under (1) above. This should give a hemicellulose which was more resistant to extraction. In a few cases cold 17 per cent sodium hydroxide was used in place of boiling 7 per cent sodium hydroxide.

In the case of vegetable ivorynut waste, one extraction with cold 5 per cent sodium hydroxide solution for 48 hours seemed to remove most of the hemicellulose. Very little hemicellulose was obtained by extracting the residue with 5 per cent sodium hydroxide solution in the boiling water bath.

Though spruce sawdust gave a fairly high percentage of carbon dioxide, the yield of hemicellulose by the method described above was very small. As a result hemicellulose from this material was not examined. It is possible that the hemicelluloses from soft woods are more soluble than those from hard woods.

TABLE I

Results of the Carbon Dioxide Determination

Sample No.	Material	CO_2 present
		per cent
1	α-Cellulose	0.06
2	Cotton fiber, untreated	0.34
3	"　　extracted with 0.5 per cent $(NH_4)_2C_2O_4$ in boiling water bath	0.20
4	Cotton extracted with 0.3 per cent NaOH in boiling water bath	0.15
5	Crystalline dextrose	0.18
6	White birch sawdust, 80 mesh	1.22
7	"　　"　　"　　extracted with 0.5 per cent $(NH_4)_2C_2O_4$	1.12
8	White birch extracted once by boiling 1 per cent NaOH.	0.68
9	"　　"　　"　　twice "　　"　　1 "　　"　　"	0.64
10	"　　"　　"　　with 0.5 per cent $(NH_4)_2C_2O_4$ and cold 5 per cent NaOH	0.72
11	White birch extracted by 7 per cent NaOH in boiling water bath	0.30
12	Spruce sawdust, 80 mesh	1.00
13	"　　extracted by 0.5 per cent $(NH_4)_2C_2O_4$	0.83
14	"　　"　　" 1 per cent NaOH	0.53
15	"　　"　　" 0.5 per cent $(NH_4)_2C_2O_4$ and cold 5 per cent NaOH	0.68
16	Spruce extracted by 7 per cent NaOH in boiling water bath	0.45
17	Catalpa sawdust, 80 mesh	1.44
18	"　　extracted by 0.5 per cent $(NH_4)_2C_2O_4$	1.07
19	Western larch, 80 mesh	0.71
20	Cottonseed hull bran	1.60
21	"　　"　　" extracted by 0.5 per cent $(NH_4)_2C_2O_4$	1.45
22	Cottonseed hull bran extracted by 0.5 per cent $(NH_4)_2C_2O_4$ and cold 5 per cent NaOH	1.12
23	Cottonseed hull bran extracted by 1 per cent NaOH	1.04
24	"　　"　　"　　"　　" 7 "　　"　　" in boiling water bath	0.30
25	Corn-cobs	1.62
26	"　　extracted by 0.5 per cent $(NH_4)_2C_2O_4$	1.49
27	"　　"　　" 0.5 "　　"　　" and cold 5 per cent NaOH	0.53
28	Corn-cobs extracted by 1 per cent NaOH	0.44
29	"　　"　　" 7 "　　"　　"	0.17

TABLE I—*Concluded*

Sample No.	Material	CO₂ present
		per cent
30	Vegetable ivorynut waste	0.30
31	" " " extracted by 0.5 per cent (NH₄)₂C₂O₄	0.38
32	Vegetable ivorynut waste extracted by 0.5 per cent (NH₄)₂C₂O₄ and 5 per cent NaOH	0.12
33	Hickory sawdust, 80 mesh	1.25
34	" extracted by 0.5 per cent (NH₄)₂C₂O₄	1.10
35	Flaxseed mucilage, crude	7.04
36	Hemicellulose from untreated white birch............	3.35
37	" " white birch previously extracted by 0.5 per cent (NH₄)₂C₂O₄...........................	2.90
38	Hemicellulose from white birch previously extracted by boiling 1 per cent NaOH. Hemicellulose was taken out by 7 per cent NaOH in boiling water bath........	2.44
39	Hemicellulose from untreated cottonseed hull bran......	3.00
40	" " cottonseed hull bran previously extracted by 0.5 per cent (NH₄)₂C₂O₄.................	2.35
41	Hemicellulose from cottonseed hull bran previously extracted twice by cold 5 per cent NaOH. Hemicellulose was taken out by 7 per cent NaOH in boiling water bath...	2.36
42	Hemicellulose from untreated corn-cobs...............	1.92
43	" " corn-cobs previously extracted by 0.5 per cent (NH₄)₂C₂O₄...........................	1.41
44	Hemicellulose from corn-cobs previously extracted by cold 5 per cent NaOH. Hemicellulose was taken out by 7 per cent NaOH in boiling water bath...........	1.05
45	Hemicellulose from corn-cobs previously extracted by cold 5 per cent NaOH. Hemicellulose was taken out by 17 " " "	0.95
46	Hemicellulose from vegetable ivorynut waste previously extracted by 0.5 per cent (NH₄)₂C₂O₄	0.36
47	Barium salt of the aldobionic acid from hydrolysis of hemicellulose from cottonseed hull bran Found...	10.18
	Theory for Ba salt of hexose aldobionic acid.......	10.40

The naphthoresorcinol test for uronic acids was made on calcium salts prepared from the following materials and in all cases found to be positive; (1) white birch and spruce sawdust, (2) cottonseed

hull bran, (3) corn-cobs, and (4) hemicelluloses prepared from all of the above materials both before and after extraction with 0.5 per cent ammonium oxalate solution.   The calcium salts were prepared by the following general method.   The material was mixed with 6 to 8 times its weight of 4 per cent sulfuric acid and heated in a boiling water bath for 15 hours.   The solution was filtered from insoluble material, neutralized by calcium carbonate, the solution filtered from the calcium sulfate, and the filtrate concentrated under reduced pressure to a small volume.   After standing, the solution was again filtered from calcium sulfate.   The calcium salts were then precipitated by a large volume of 95 per cent ethanol and finally rubbed until they became granular.   In some cases the barium salts were prepared by the same general method.

In the case of vegetable ivorynut waste both the hemicellulose and the insoluble material remaining after extraction with sodium hydroxide solution gave a bluish fluorescent ether solution by reflected light in place of the regular naphthoresorcinol test for a uronic acid.   The indication was that no uronic acid was present. Unfortunately no salts were prepared from this material.

Discussion of Experimental Results and Summary

A study of the experimental results leads to certain fairly definite conclusions among which are the following:

1. Determination of the carbon dioxide evolved by plant materials when heated with 12 per cent hydrochloric acid according to the method of Lefèvre and Tollens yields valuable information both qualitative and quantitative relative to the presence of uronic acids.

2. While many hemicelluloses contain uronic acids and are thus uronides of the sugars, others, such as that from vegetable ivorynuts, contain no uronic acid but are true polysaccharides.

3. Some plant materials, such as white birch sawdust and corn-cobs, apparently contain two or more hemicelluloses, which may be isolated by variation in the method of extraction.   Other plant materials, such as cottonseed hulls, apparently contain but a single hemicellulose.

4. Partial hydrolysis of some of the hemicelluloses isolated above yielded reducing sugars together with aldobionic acids similar to those isolated from some of the plant gums.   One such

aldobionic acid from cottonseed hull hemicellulose was isolated as the barium salt. It thus appears that some of the uronic acid hemicelluloses are similar in their general structure to some of the plant gums.

5. It is possible that the various hemicelluloses may yield approximately the same percentage carbon dioxide when heated with 12 per cent hydrochloric acid. If this is true, the ratio between the percentage carbon dioxide in the hemicellulose and 100 per cent will be approximately the same in all cases and an average factor will be found which when multiplied by the percentage carbon dioxide in the pectin-free material will yield the percentage of uronic acid hemicellulose present. For example the pectin-free hemicellulose obtained from white birch, Sample 37 in Table I, gave 2.9 per cent carbon dioxide. In this material the ratio between the per cent of carbon dioxide and 100 per cent is 34.5. When pectin-free white birch sawdust containing 1.12 per cent carbon dioxide, Sample 7 in Table I, is extracted with 1 per cent sodium hydroxide, the resulting sawdust, Sample 8, contains 0.68 per cent carbon dioxide. Alkali extraction thus caused a decrease of 0.44 per cent in the carbon dioxide content. If this 0.44 per cent is multiplied by the factor 34.5, the product, which is approximately 15, should be the percentage of uronic acid hemicellulose that is removed from the sawdust by 1 per cent sodium hydroxide. It is known that approximately 20 per cent of white birch sawdust is soluble in boiling 1 per cent sodium hydroxide. It thus appears that other materials than uronic acid hemicelluloses are dissolved out of this wood by boiling 1 per cent sodium hydroxide.

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OXIDATION AND REDUCTION RELATIONS BETWEEN SUBSTRATE AND PRODUCTS IN THE ACETONE-BUTYL ALCOHOL FERMENTATION*

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The anaerobic fermentation of a carbohydrate or related compound is primarily a process of oxidation and reduction. All products of sugar fermentation are formed by a series of intermolecular and intramolecular oxidations and reductions, with attendant coupling and splitting reactions. Complex as these rearrangements may be, there are certain quantitative relationships which must always hold true. If the fermentation is anaerobic, oxidation of one compound must be accompanied by reduction of another compound. The various oxidized and reduced fermentation products therefore, must always be present in a ratio such that the degree of oxidation of the system as a whole remains constant. This relationship, is, of course, entirely independent of the fermentative mechanism.

The following is a convenient method of calculating such an oxidation-reduction balance. Let us take as a standard of the degree of oxidation a compound in which hydrogen and oxygen are present in equivalent proportions, that is, in the ratio to form water. Examples of such compounds are glucose ($C_6H_{12}O_6$), methyl glyoxal ($C_3H_4O_2$), and acetic acid ($C_2H_4O_2$). Then we may evaluate other compounds according to the number of hydrogen atoms they contain in excess of the number necessary to combine with the oxygen present in the molecule. Thus acetaldehyde (C_2H_4O) has a reduction value of $+1$, since it contains 2 atoms of hydrogen more than is needed to combine with the

oxygen present. On the same basis, succinic acid ($C_4H_6O_4$) has a value of -1, and carbon dioxide (CO_2) has a reduction value of -2. A convenient method of drawing up an oxidation-reduction balance for a fermentation is illustrated in Table I. The data are taken from Braak (1) p. 94. It will be noted that for consistency in algebraic representation, a compound fermented appears with minus sign.

The oxidation-reduction balance, as well as the carbon balance, for this fermentation, shows that the products were accurately determined, and that there was present no considerable quantity of any product not determined.

TABLE I

Fermentation of Glycerol by Bacterium aerogenes

Compound	Mols of products produced	Reduction value	$-H_2$	$+H_2$	Mols carbon	$-C$	$+C$
			mols	*mols*		*mols*	*mols*
Glycerol	-0.090	$+1$	0.090		-0.270	0.270	
Carbon dioxide	$+0.057$	-2	0.114		0.057		0.057
Hydrogen	$+0.062$	$+1$		0.062			
Formic acid	$+0.012$	-1	0.012		0.012		0.012
Acetic "	$+0.007$	0			0.014		0.014
Lactic "	$+0.003$	0			0.009		0.009
Succinic acid	$+0.006$	-1	0.006		0.024		0.024
Ethyl alcohol	$+0.081$	$+2$		0.162	0.162		0.162
Total			0.222	0.224		0.270	0.278

In some fermentations it is often difficult to make reliable sugar determinations. The setting up of a carbon balance is then impossible. Since a carbohydrate has a reduction value of zero, an oxidation-reduction balance may be made without data on sugar concentration.

The application of oxidation-reduction balances to many fermentation tables given in the literature yields a number of interesting results. In some cases, even where the carbon balance approaches 100 per cent, the oxidation-reduction balance reveals large errors in analysis. It may be concluded that, in fermentation work, the balancing of the oxidation and reduction products is as worthy of attention as the balancing of carbon.

It is evident from the foregoing, that in a given fermentation, if we substitute for the compound being fermented another compound more oxidized or more reduced, we must of necessity change the nature of the fermentation products, or the ratio in which they are formed. Suppose, in any anaerobic fermentation, that we substitute mannitol for glucose. The products of the mannitol fermentation, taken as a whole, must be more reduced than the products of the glucose fermentation. If a more oxidized substrate than glucose, such as gluconic acid, is fermented, the products of fermentation must be more oxidized than the products of the glucose fermentation. In a fermentation which yields a number of products, there are many ways in which this may be accomplished. The manner in which the changes necessary to preserve the oxidation-reduction balance actually take place, however, is dependent upon the mechanism of the fermentation.

A study of the changes in the ratio in which the various fermentation products occur when different substrates are fermented is of especial interest in the case of fermentations carried on by *Clostridium acetobutylicum*, because of the variety of fermentation products, and because of the large number of compounds fermentable by this organism.

<center>EXPERIMENTAL</center>

Procedure and Methods—The fermentation experiments to be described were carried out with a strain of *Clostridium acetobutylicum*, the organism used in the industrial manufacture of acetone and butyl alcohol. The medium used was the peptone-salt solution described by Speakman (2), except that distilled water was replaced by lake water. Inoculation was made from a vigorous 24 hour culture of the organism in 6 per cent corn mash. 1 per cent of inoculum was used in every case, and the fermentations were incubated at 37°. Although no particular precautions were used to insure anaerobic conditions, subsequent experiments showed that the amount of inoculum added was amply sufficient to bring the oxidation-reduction potential of the medium down to the low potential necessary to initiate fermentation. At the end of each large fermentation, the medium was tested for the presence of foreign organisms, but none was found.

The series of fermentations carried out were of two types. In

the first series only an incomplete analysis was made, the purpose being to determine the ratio in which the "solvents" (acetone, ethyl alcohol, and butyl alcohol) were produced. In these fermentations, the analytical procedure was as follows: A known volume of culture was placed in a distilling flask, and $\frac{1}{2}$ of its volume was distilled into a volumetric flask. Total solvents were determined on the distillate by the pycnometer method. Acetone in the distillate was determined by Goodwin's (3) iodoform method. The distillate was then saturated with NaCl and redistilled. The solvents were salted out of this second distillate by the addition of anhydrous K_2CO_3. Ethyl alcohol was determined in the solvent mixture by Bogin's (4) water titration method. Butyl alcohol was determined by difference. Sugar determinations were made by the micro method of Stiles, Peterson, and Fred (5).

In the second series of fermentations, fairly complete analyses were made at intervals throughout the fermentation. The fermentations were set up as follows: A large flask (6 liter or 10 liter capacity) filled to the neck with medium, was inoculated and tightly closed with a rubber stopper. Directly below the stopper was placed a glass wool plug which proved very efficient in breaking up froth produced by the fermentation. The stopper was fitted with a glass tube leading to a gas meter. This tube was fitted with a 3-way glass stop-cock so arranged that the gas flow could be diverted from the meter into a gas sampling tube. The sampling tube was arranged to collect a sample over mercury at a constant pressure slightly above atmospheric. The stopper of the fermentation flask was also fitted with a specially constructed pipette by means of which a liquid sample could be taken at intervals, under sterile conditions, and without permitting the escape of gas. The stopper was also fitted with a platinum electrode and a saturated KCl bridge, for the taking of oxidation-reduction potentials at intervals during the fermentation. The tip of the KCl tube contained a wad of fine, parallel, asbestos fibers, fused into the glass. It was found that such a tip lent itself well to sterilization, and gave good conductivity with negligible diffusion.

In these large fermentations, determinations at 4 hour intervals (at 2 hour intervals early in the fermentation) were made as

follows: (1) gas meter reading; (2) analysis of gas sample for CO_2 and H_2; (3) determination in liquid sample of ethyl and butyl alcohols, acetone, acetic and butyric acids, and pH; and (4) oxidation-reduction potential reading. The gas analyses were made with Burrell and Oberfell's modified Orsat apparatus (6). The pH was determined on the liquid samples with the quinhy-

TABLE II

Fermentation of Glucose by Clostridium acetobutylicum

Duration of fermentation	Volume of culture	Glucose before fermentation	Glucose fermented	Final acidity, cc. 0.1 N in 10 cc. culture	Solvents, total and percentage distribution			
					Total	Acetone	Ethyl alcohol	Butyl alcohol
hrs.	*cc.*	*per cent*	*per cent*		*gm. per l.*	*per cent*	*per cent*	*per cent*
104	2500	2.44	95.9	3.2	8.1	25.0	16.5	58.5
104	2500	2.44	93.8		7.9	25.2		
84	2500	2.08	93.3	3.0	6.1	26.6	8.1	65.3
90	2500	1.69	92.8		4.7	30.9	9.2	58.9
157	2500	3.65	91.0	3.6	11.6	26.6	13.1	60.3
91	2500	2.62	95.0	3.0	7.9	27.0	11.9	61.1
60	2500	1.02	94.0	4.2	1.6	40.3		
60	2500	1.02	94.0	5.2	1.3	29.8		
84	2500	2.07	93.7	3.2	5.2	28.5	12.1	59.4
84	2500	2.07	93.7	3.8	5.8	27.4		
159	2500	3.05	94.0	3.0	9.4	28.4	11.70	59.9
159	2500	3.05	93.8	3.1	9.4	28.6	12.6	58.8
135	2500	3.05	94.0	3.6	9.1	27.0	13.2	59.8
193	2500	4.03	86.3	3.4	11.5	28.4	12.5	59.1
193	2500	4.03	85.1	3.3	11.3	28.6	11.9	59.5
50	2500	0.975	93.5	4.5	1.3	29.5		
50	2500	0.975	93.5	4.9	1.1	31.8	7.0	63.0
50	2500	0.975	93.5	4.3	1.3	29.1		
50	2500	0.975	93.5	4.5	1.4	28.7		
	2500	2.38	95.3		6.9	29.3	10.7	60.0
120	3000	2.50	95.2	3.2	7.55	25.4	11.3	65.3
104	10,000	2.52	94.8	1.4	7.9	29.0	13.8	57.2

drone electrode. Alcohols, volatile acids, and acetone were determined by micro methods developed especially for these fermentations, and described elsewhere (7). Carbon dioxide dissolved in the medium at various times was calculated from the solubility of CO_2 at 37°, and the partial pressure of CO_2 in the gas being evolved from the fermentation. The gas production was

also corrected for the change in volume of the culture due to the removal of liquid samples at intervals.
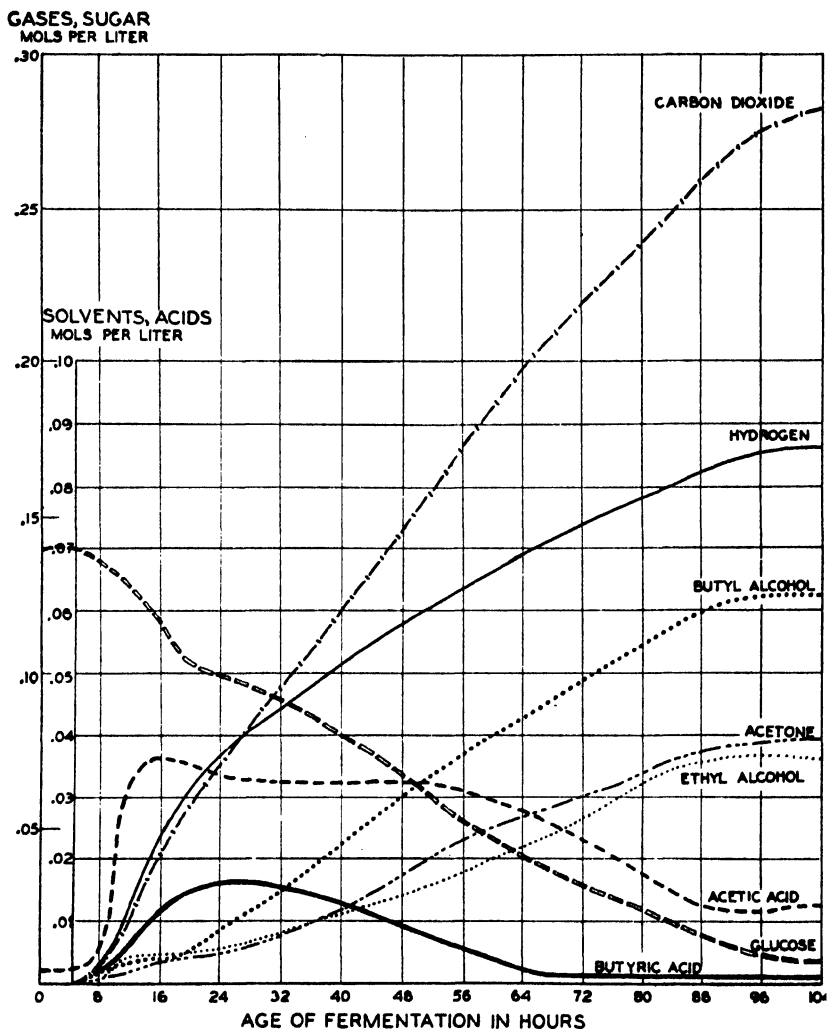
CHART 1. Fermentation of glucose by *Clostridium acetobutylicum*

Glucose Fermentation—In Table II are listed the results of analyses made on a number of glucose fermentations. These fermentations were carried out in order to determine the effect of

variation in sugar concentration on the relative yields of the various fermentation products. It will be seen that in the lower concentrations of carbohydrate, a larger percentage of the sugar was converted into acids. The ratio in which the neutral products occur, while subject to great variation, does not seem to be dependent upon sugar concentration. The low yield of ethyl alcohol from the fermentations where the initial carbohydrate concentration was 0.975 per cent is probably due to loss of alcohol in distillation, since, in this experiment, an extra distillation was necessary in order to obtain the solvents in a dry state.

In order to obtain a more complete picture of the glucose fermentation, a large fermentation was set up on which fairly com-

TABLE III

Glucose Fermentation

Age of fermentation	Carbon balance	Oxidation-reduction balance as excess H_2 evolved	Calculated CO_2*	Observed CO_2	Calculated H_2*	Observed H_2
hrs.	*per cent*	*mols per l.*	*mols per l.*	*mols per l.*	*mols per l.*	*mols per l.*
8	100.1	+0.008	0.018	0.005	0.024	0.005
16	106.1	+0.019	0.086	0.043	0.114	0.048
24	103.4	+0.022	0.107	0.072	0.122	0.075
48	102.3	+0.027	0.182	0.148	0.157	0.117
72	95.2	+0.005	0.236	0.221	0.174	0.148
104	92.9	+0.005	0.290	0.285	0.178	0.176

* See text for basis of calculations.

plete analyses were made at short intervals. The data obtained are plotted in Chart 1. The sugar determinations from which the sugar destruction curve was drawn are evidently unreliable. That fictitiously high results were obtained, due, perhaps, to the presence of some reducing intermediate compound, is shown by the fact that the carbon balance is high during the course of the fermentation, but drops off markedly toward the end. The carbon balances calculated for various times throughout the fermentation are shown in Table III (second column). The oxidation-reduction balance is found also in Table III (third column). The excess of reduced compounds present, expressed in Table III as excess hydrogen evolved, gives definite evidence of the presence during

the fermentation of an undetermined intermediate more oxidized than glucose.

In postulating fermentation mechanisms, there is a great temptation to construct an equation representing the breakdown of a definite number of sugar molecules to a definite number of molecules of each of the fermentation products. In a fermentation where a number of end-products are found, such an equation cannot adequately represent the facts. The rate of formation of the various end-products from common unstable intermediates obeys the fluctuations of a delicate dynamic equilibrium rather than the precepts of a numerical relationship expressible in a single equation. There are, however, certain experimentally determined quantitative relationships which reveal the skeletal structure, but not the details, of the mechanism of the formation of the various products. These relationships are conveniently expressible by a set of equations representing the ratios which exist between the quantities of gases evolved and the quantities of other products formed. In order to express these ratios as balanced equations, it is necessary to write an equation as if 1 definite molecule of glucose gave rise to one definite end-product, whereas in reality such is undoubtedly not the case. The equations are the following.

(1)	$C_6H_{12}O_6$	$= 2C_2H_5OH + 2CO_2$
(2)	$C_6H_{12}O_6$	$= C_4H_9OH + 2CO_2 + H_2O$
(3)	$C_6H_{12}O_6 + H_2O$	$= CH_3\,CO\,CH_3 + 3CO_2 + 4H_2$
(4)	$C_6H_{12}O_6 + 2H_2O$	$= 2CH_3COOH + 2CO_2 + 4H_2$
(5)	$C_6H_{12}O_6$	$= C_3H_7COOH + 2CO_2 + 2H_2$
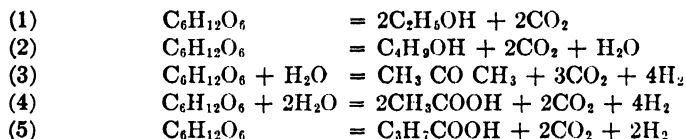
It should be noted that these equations are not intended to mean that certain definite molecules of glucose are converted exclusively to one end-product. They are rather a statement that the stoichiometric relations involved in the conversion of the common intermediates to the various end-products are such that the quantitative relationships connecting glucose destroyed, gases evolved, and solvents and acids produced are the same as those which would obtain if the equations were a literal statement of fact.

The various mechanisms which have been suggested for this fermentation have all been tailored to fit the relationships expressed

by the above equations, and are all, therefore, equally substantiated by quantitative data in accord with the equations. The mechanisms proposed by Reilly *et al.* (8), Neuberg and Arinstein (9), Donker (10), and Schoen (11) are, in the main, the same. These investigators consider that the glucose molecule splits into 2 molecules of methyl glyoxal, which is subsequently broken down to acetaldehyde and formic acid, or through pyruvic acid to acetaldehyde and carbon dioxide. The formic acid gives rise to carbon dioxide and hydrogen, while the acetaldehyde, by oxidations, reductions, and condensations, gives rise to the other products. Acetone is considered to be formed by the decarboxylation of acetoacetic acid.

TABLE IV

Procedure Followed in Calculating the Theoretical Production of Carbon Dioxide and Hydrogen

Product	Amount produced*	Theoretical carbon dioxide production	Theoretical hydrogen production
	mols per l.	*mols per l.*	*mols per l.*
Acetic acid......................	0.010	0.010	0.020
Butyric "	0.001	0.002	0.002
Ethyl alcohol....................	0.037	0.037	
Butyl "	0.062	0.124	
Acetone.........................	0.039	0.117	0.156
Total.........................		0.290	0.178
Observed gas production...................		0.285	0.176

* Amount present minus amount introduced in inoculum.

The application of the equations given above to the data of the present glucose fermentation enables us to calculate, from the amounts of acids and solvents present, the volumes of carbon dioxide and hydrogen which should have been evolved in their production. In Table III, such computed values are compared with the observed values. As an example of the method followed, the detailed calculations for the last set of values in Table III are given in Table IV.

It will be seen that while at the end of the fermentation there is substantial agreement, during the early part of the fermentation the evolved gases are not nearly sufficient to account for the acids

and solvents produced. This is further evidence of the existence of an intermediate product, which is a precursor of hydrogen (either molecular hydrogen or hydrogen available for reduction) and carbon dioxide. The minute amounts of formic acid known to be present are not nearly great enough to account for the discrepancy. However, none of the mechanisms proposed for the fermentation involves a precursor of carbon dioxide and hydrogen other than formic acid.

TABLE V

Fermentation of Mannitol by Clostridium acetobutylicum

Duration of fermentation	Volume of culture	Mannitol before fermentation	Final acidity, cc. 0.1 N in 10 cc. culture	Solvents, total and percentage distribution			
				Total	Acetone	Ethyl alcohol	Butyl alcohol
hrs.	*cc.*	*per cent*		*gm. per l.*	*per cent*	*per cent*	*per cent*
260	500	2.50		7.6	11.1		
260	500	2.50		8.2	10.9		
260	500	2.50	3.7	7.1	10.6	6.5	82.9
260	500	2.50		7.2	10.1		
	500	2.50		8.5	9.4		
	500	2.50		7.2	9.25		
	500	2.50		8.7	10.1	10.0	80.4
	500	2.50		8.2	9.7		
234	2500	2.50	4.3	7.7	13.5	3.7	82.8
222	2500	2.46	4.8	7.7	12.1	5.8	62.1
200	2500	2.46	4.4	7.9	12.8	4.6	82.6
256	2500	2.46	4.6	7.3	12.6	5.3	82.1
175	2500	2.46	4.2	8.0	12.2	4.6	83.2
	2500	2.50		8.2	11.6	7.4	81.0
96	10,000	2.50	5.5	6.6	9.6	8.7	81.7

The fundamental validity of the five equations given above, is, however, indicated by the fact that good agreement between observed and calculated gas production is obtained at the end of the fermentation. It will be seen from Table II that the discrepancies in the oxidation-reduction balance, and in the gas production, are much too large, particularly early in the fermentation, to be the result of experimental error in the determinations which were made.

Mannitol Fermentation—In Table V, analyses of a number of mannitol fermentations are given. The figures show that in the

mannitol fermentation the distribution of solvents differs greatly from the distribution found in the glucose fermentation.   There
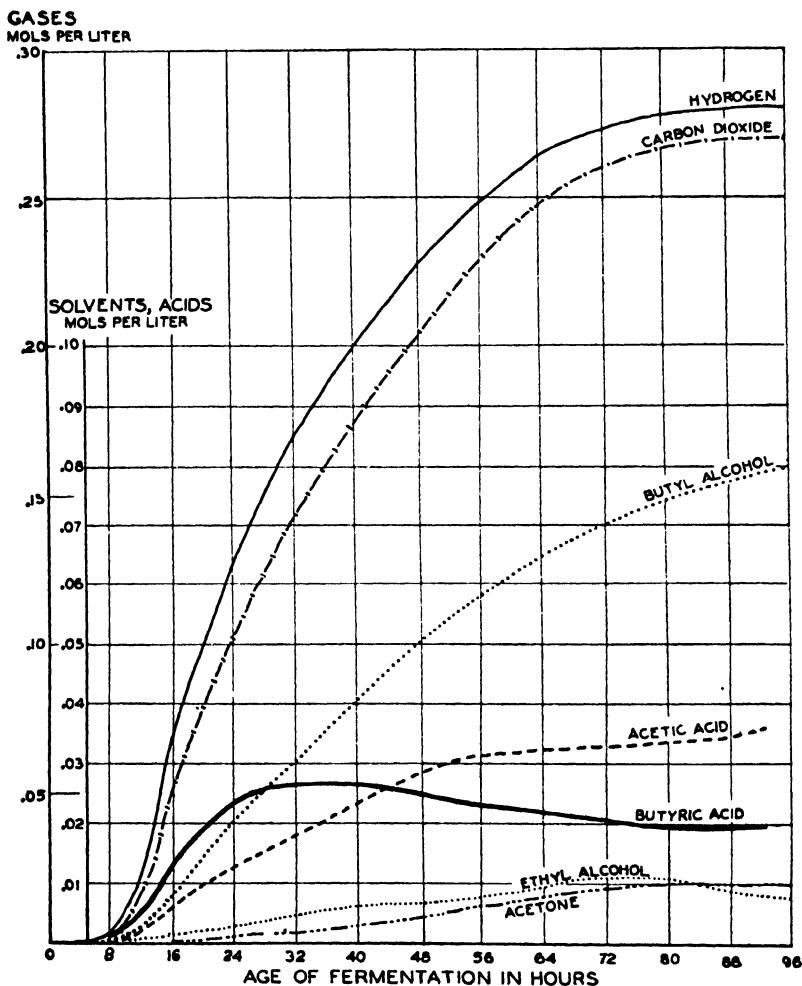
CHART 2. Fermentation of mannitol by *Clostridium acetobutylicum*

is an increased production of butyl alcohol, and a corresponding decrease in acetone production.   It is evident that the superabundance of available hydrogen present in the fermentation of

this reduced compound appears, to some extent at least, as an increased proportion of reduced compounds in the solvents produced.

Chart 2 represents a mannitol fermentation upon which the main fermentation products were determined at short intervals. The initial mannitol concentration was 25 gm. per liter.

The changes in the fermentation brought about by the fermentation of a reduced compound are evident when Chart 2 is compared with the chart representing the glucose fermentation. Hydrogen production is much greater; acetone, whose formation probably involves only oxidations and decarboxylations, is much less; the volatile acid contains a larger percentage of butyric acid, and the production of butyl alcohol is greatly stimulated. The

TABLE VI

Mannitol Fermentation

Age of fermentation	Oxidation-reduction balance as excess H_2 evolved	Calculated CO_2	Observed CO_2	Calculated H_2	Observed H_2
hrs.	*mols per l.*	*mols per l.*	*mols per l.*	*mols per l.*	*mols per l.*
12	+0.007	0.020	0.016	0.027	0.023
16	+0.003	0.052	0.051	0.067	0.067
24	+0.006	0.108	0.103	0.131	0.128
48	−0.005	0.198	0.204	0.220	0.227
72	−0.009	0.254	0.260	0.268	0.273
96	+0.002	0.273	0.270	0.285	0.280

amount of ethyl alcohol produced, however, is less. Other experiments have indicated that ethyl alcohol production is independent of the degree of oxidation or reduction of the substrate. If we calculate from the carbon present in the form of fermentation products the amount of mannitol fermented, we are enabled to draw up oxidation-reduction balances for various stages of the fermentation. Such balances are listed in Table VI. It will be seen that the large discrepancies found in the oxidation-reduction balances of the glucose fermentation are absent. This evidence indicates the absence of an oxidized intermediate compound.

If we make the reasonable assumption that mannitol and glucose are broken down in much the same way, then the quantitative relationships which obtain in the mannitol fermentation are the

same as those expressed in the five equations given for the glucose fermentation, except that 1 additional molecule of hydrogen is evolved for every molecule of mannitol fermented. In Table VI, gas production calculated on these assumptions is compared with the observed gas production. The agreement is fairly good throughout the fermentation. There is no definite evidence of the presence of a precursor of carbon dioxide and hydrogen.

We may conclude that in general the mannitol and glucose fermentative mechanisms are similar, but that, because of the reduced character of the mannitol molecule, 2 additional atoms of hydrogen are available. This results in an increased production of fermentation products whose formation involves reduction reactions.

Calcium Gluconate Fermentation—Since gluconic acid is an oxidation product of glucose, we should expect a gluconic acid fermentation to result in products more oxidized than those of the glucose fermentation. It is impossible to ferment a $2\frac{1}{2}$ per cent solution of gluconic acid, since the pH of such a solution is very low. It was found also that the lactone of gluconic acid was so easily hydrolyzed that it could not be used. Calcium gluconate is readily fermentable. A calcium gluconate fermentation, however, is affected by the neutralizing action of the calcium which is liberated when a molecule of calcium gluconate is fermented. Donker (10), Van der Lek (12), and others have found that the addition of calcium carbonate to a glucose fermentation results in an accumulation of acids, and checks their conversion into solvents. A similar result is to be expected in the fermentation of calcium gluconate. A calcium gluconate fermentation in which the liberated calcium was neutralized by the addition of sulfuric acid at intervals was found to be slow and irregular. It was also impossible to determine at what rate the sulfuric acid should be added.

Chart 3 illustrates a calcium gluconate fermentation upon which analyses were made at short intervals. The initial concentration of calcium gluconate was 2.98 per cent (equimolecular with a $2\frac{1}{2}$ per cent glucose concentration). It is evident that acid production has been enormously stimulated by the calcium liberated from the fermented calcium gluconate.

Among the solvents, acetone was produced in much larger

quantities than the alcohols. This preponderance of acetone is
to be expected from the fermentation of an oxidized substrate.
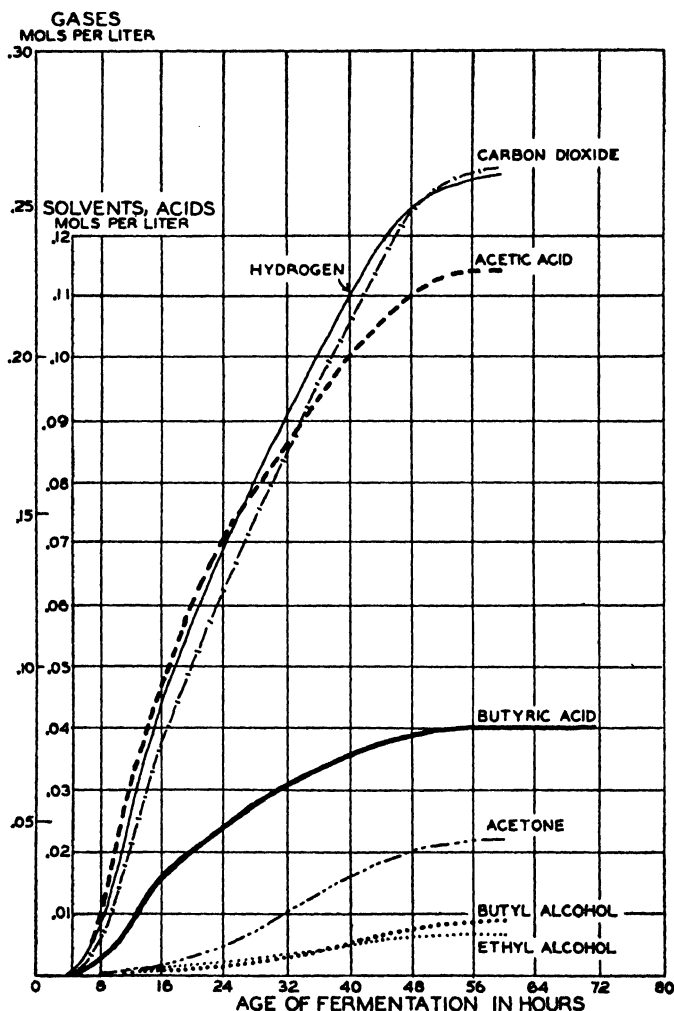
CHART 3. Fermentation of calcium gluconate by *Clostridium acetobutylicum*

A decreased production of hydrogen should also be expected.
This, however, is masked by the stimulation of hydrogen produc-
tion due to the large amounts of acids produced. We have, in

calcium gluconate, two factors influencing the ratio of products, the oxidized character of the substrate, and the excess of base liberated in the fermentation.

If we subtract 1 molecule of hydrogen from—and add 1 molecule of water to—the right side of each of the five equations given for the glucose fermentation, and preserve the balance of the equations by substituting gluconic acid for glucose on the left side of each equation, the result may be expected to represent the gluconic acid fermentation. Table VII contains comparisons of values for carbon dioxide and hydrogen calculated on this basis with the observed values. The oxidation-reduction balances found in Table VII were calculated in a manner analogous

TABLE VII

Calcium Gluconate Fermentation

Age of fermentation	Oxidation-reduction balance as excess H₂ evolved	Calculated CO₂	Observed CO₂	Calculated H₂	Observed H₂
hrs.	*mols per l.*	*mols per l.*	*mols per l.*	*mols per l.*	*mols per l.*
12	−0.007	0.048	0.042	0.048	0.049
24	+0.024	0.136	0.123	0.140	0.137
36	+0.032	0.209	0.188	0.204	0.198
48	+0.050	0.268	0.245	0.253	0.248
60	+0.038	0.285	0.261	0.265	0.261

to that used in calculating oxidation-reduction balances for the mannitol fermentation.

While the observed and calculated values for hydrogen production agree well, there is definite evidence of the existence of undetermined carbon dioxide. This shortage of carbon dioxide is, of course, reflected also in the oxidation-reduction balance. If this deficit in evolved carbon dioxide were due to an intermediate compound, that compound must be a precursor of carbon dioxide, but not of hydrogen, since there is no appreciable quantity of unaccounted for hydrogen. Acetoacetic acid is such a precursor of carbon dioxide. If the discrepancy is due to the fact that the acetone of this fermentation was present largely as acetoacetic acid, it should be possible to demonstrate the presence of this acid in the culture after the fermentation.

At the end of the fermentation, the culture gave distinctly positive results when tested qualitatively for the presence of acetoacetic acid by the method of Bondi and Schwarz (13) and of Harding and Ruttan (14). Quantitative determination by the aspiration method showed the presence in the culture of 0.34 gm. of acetoacetic acid per liter, after 12 hours of vigorous aspiration. Another calcium gluconate fermentation also gave positive qualitative and quantitative tests for acetoacetic acid. It is reasonable to suppose that the existence of considerable quantities of acetoacetic acid in this fermentation is due to excess of base present, which fixes a part of the acetoacetic acid as its calcium salt. The amount of acetoacetic acid found, however, is not sufficient to account for the difference between observed and calculated carbon dioxide production.

Arabinose Fermentation—The mechanism of the breakdown of a pentose must necessarily be different from the mechanism of the fermentation of a six-carbon compound. The hexose mechanism is such that 2 molecules of carbon dioxide arise from each molecule of sugar, excepting the case of acetone formation, where 3 molecules of carbon dioxide are evolved. The evolution of a molecule of carbon dioxide is an oxidative process giving rise to 4 atoms of hydrogen, available for evolution or for reduction reactions. If the mechanism of the pentose fermentation were such that 2 molecules of carbon dioxide were evolved from each pentose molecule, the resulting 8 atoms of hydrogen would be distributed among only 3 carbon atoms; whereas, in the hexose fermentation the corresponding 8 atoms of hydrogen are distributed among 4 carbon atoms. This would make the pentose fermentation a reduced fermentation, resembling the mannitol fermentation. If, on the other hand, only 1 molecule of carbon dioxide were split out of the pentose molecule, there would result only 4 atoms of hydrogen for the reduction of the remaining 4 carbon atoms. The result would be an oxidized fermentation, resembling the gluconic acid fermentation.

Table VIII gives the results of a number of preliminary arabinose fermentations. It will be seen that, compared with a glucose fermentation, the percentage of acetone in the solvents produced has increased markedly, at the expense of the butyl alcohol. This is evidence of an oxidized fermentation, since acetone formation is

strictly a process of oxidation and decarboxylation, while butyl alcohol is the end-product of a series of reactions involving reduction.

An arabinose fermentation upon which a more complete analysis was made is shown in Chart 4. Here there are abundant evidences of an oxidized type of fermentation. Besides a high yield of acetone, there is a higher ratio of acetic to butyric acid than is obtained in the glucose fermentation. The amount of hydrogen produced is also less.

The irregularity of the sugar destruction curve implies the presence of a highly reducing intermediate. The oxidation and

TABLE VIII

Fermentation of Arabinose by Clostridium acetobutylicum

Duration of fermentation	Volume of culture	Arabinose before fermentation	Arabinose fermented	Final acidity, cc. 0.1 N in 10 cc. culture	Solvents, total and percentage of distribution			
					Total	Acetone	Ethyl alcohol	Butyl alcohol
hrs.	*cc.*	*per cent*	*per cent*		*gm. per l.*	*per cent*	*per cent*	*per cent*
104	750	2.10	92.3	5.2	4.8	35.9		
	750	2.10	81.0		4.7	41.3		
	1500	2.62	73.2		6.9	40.3	13.7	46.0
140*	500	2.62	90.9	4.4	8.4	33.8	8.8	56.3
140*	500	2.62	95.5	3.8	9.2	35.9		
68	5900	2.47	94.5	3.2	8.7	41.4	17.5	41.1

* The medium used in these two fermentations consisted of lake water, phosphates, and arabinose.

reduction balance also gives evidence of an intermediate, more oxidized than a carbohydrate, which exists in the fermentation in appreciable quantities, and disappears only at the end of the fermentation. This oxidation-reduction balance, calculated as excess hydrogen evolved in mols per liter, is at 12 hours, +0.034; at 24 hours, +0.031; at 36 hours, +0.029; at 48 hours, +0.013; and at 68 hours, −0.008

The oxidized character of this fermentation is evidence that the tendency is toward the splitting out of only 1 molecule of carbon dioxide from each arabinose molecule. The total amount of carbon dioxide evolved by the fermentation, however, shows that more than 1 molecule of carbon dioxide was split out of each arabi-

nose molecule.   The total CO_2 evolution is 0.264 mols per liter of culture.   Of this, 0.062 mols, calculated from the acetone produc-

GASES, SUGAR
MOLS PER LITER
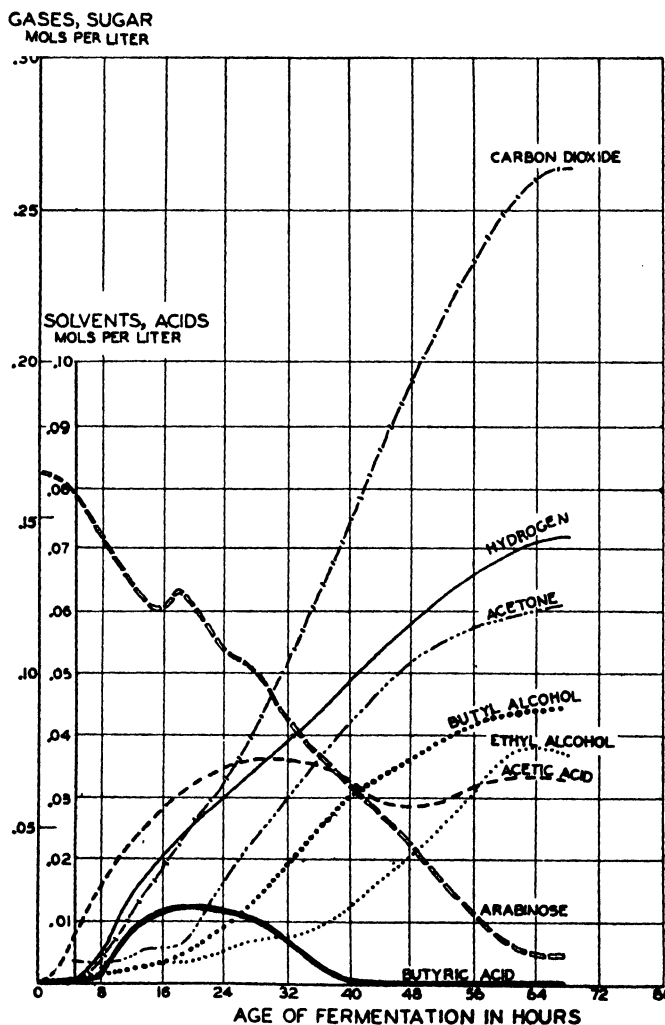
CHART 4. Fermentation of arabinose by *Clostridium acetobutylicum*

tion, is the result of the decarboxylation of acetoacetic acid.   The remainder is 0.202 mols, while the number of two-carbon units produced by the fermentation is 0.283 mols.   By a two-carbon

unit is meant a molecule of a two-carbon product or a half mole-
cule of a four-carbon product, acetone being considered derived
from a four-carbon product.   There are then 1.4 two-carbon units
per mol of "primary" carbon dioxide.   By "primary" carbon
dioxide is meant total carbon dioxide minus carbon dioxide arising
from the decarboxylation of acetoacetic acid.   A hexose type of
fermentation would yield a quotient of 1.0, while a quotient of
2.0 might be expected from a pentose fermentation.

Van der Lek (12), who studied the fermentation of arabinose by
a slightly different strain of *Clostridium acetobutylicum*, obtained a
quotient of 1.25.   Van der Lek considers that the fermentation
of arabinose takes two courses, part of the sugar being fermented
as a pentose, *i.e.* with the evolution of 1 molecule of "primary"
carbon dioxide per molecule of arabinose, and part being, prior
to fermentation, transformed by the cell into a hexose or hexose
polymer, the cell using for this puropse the same mechanism by
which it synthesizes granulose from a pentose.   This cell-formed
hexose is fermented with a production of 2 molecules of carbon
dioxide per hexose molecule.

An alternative explanation is entirely possible.   Let us assume
that the primary splitting of the arabinose molecule results in a
two-carbon and a three-carbon compound.   The three-carbon
compound will be methyl glyoxal or an isomer of it, and the two-
carbon compound will be an isomer of acetic acid, *e.g.* glycolic alde-
hyde.   Indirect evidence that such is the cleavage is afforded by
the *Lactobacillus pentoaceticus* fermentation, where arabinose is
broken down into acetic and lactic acids in equimolecular propor-
tions.   The second step in the fermentation would then be a
breakdown of the three-carbon compound into carbon dioxide, a
two-carbon compound, and hydrogen available for reduction
reactions.   The amount of hydrogen available for reduction would
be, however, only half the amount available in a hexose fermenta-
tion.   There would therefore be a tendency to relieve this paucity
of hydrogen by the oxidation of compounds which would not be
oxidized in the hexose fermentation.   For instance, the glycolic
aldehyde produced might have the choice of rearrangement to
acetic acid or oxidation to carbon dioxide through glyoxal or
glyoxylic acid.   Lebedev (15) has found that a yeast enzyme
preparation is capable of fermenting glyoxylic acid with the

production of acetaldehyde and carbon dioxide. This is an example of the type of reaction which may take place in the arabinose fermentation. Glyoxylic acid, like pyruvic acid, is an
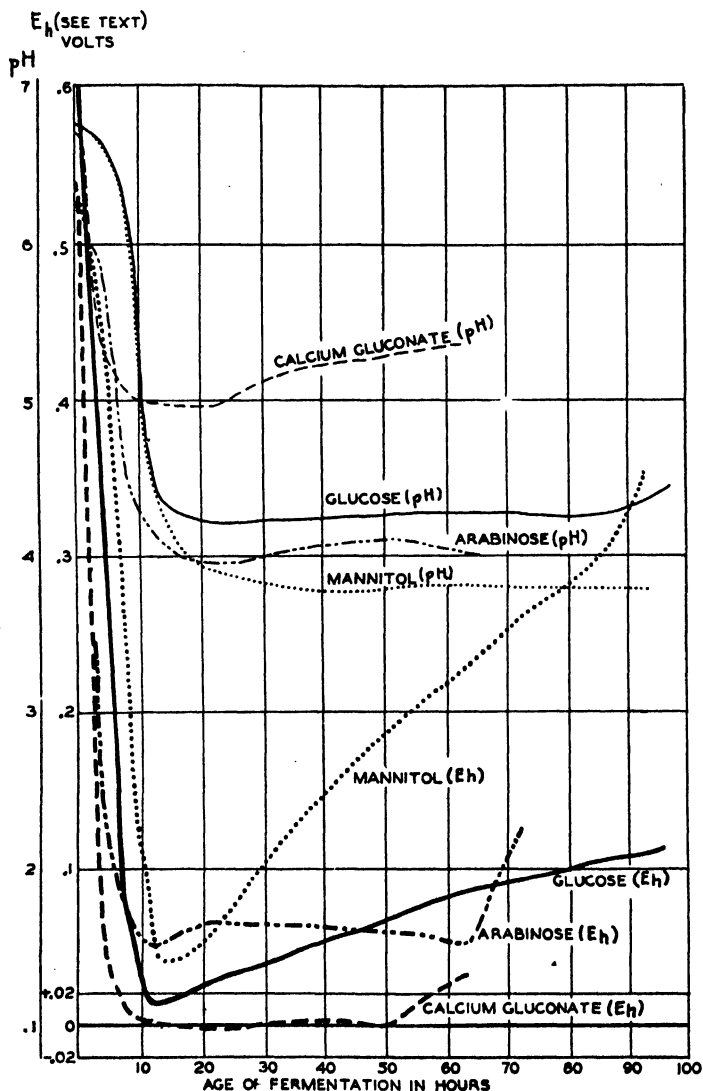
CHART 5. Hydrogen ion concentration (pH) and oxidation-reduction potential (E_h) of media during fermentation.

α-keto acid, and might be easily decarboxylated by bacterial enzymes.

Whatever the mechanism of the arabinose fermentation may be, it is evident that there exists some mechanism by which carbon dioxide in excess of 1 molecule per arabinose molecule may be evolved. That this excess carbon dioxide is formed only with difficulty is evidenced by the oxidized character of the arabinose fermentation.

Oxidation-Reduction Potentials—On Chart 5 are plotted pH and oxidation-reduction potential data for the fermentation of the compounds mentioned. With the exception of the glucose fermentation, the curves of Chart 5 were obtained from the same flasks as were used in obtaining the data of Charts 1 to 4. An attempt has been made to render the E_h data for the various fermentations comparable by plotting, not the observed potentials, but the potentials which would have existed between the bright platinum electrode and a hydrogen electrode immersed in the same solution. In other words, the E_h values are calculated to a pH of zero, "ideal" conditions being assumed, *i.e.* that the electrode behaves as a hydrogen electrode under a definite hydrogen pressure so that $- \dfrac{dE}{dpH} = 0.0615$ at $37°$.

It will be seen by comparison with Charts 1 to 4, that in every case, the preliminary rapid fall in potential is coincident with the induction period of the fermentation. The behavior of the electrode during the course of the fermentation varies with the substrate fermented. In the calcium gluconate fermentation, it has the potential of a hydrogen electrode until the end of the active fermentation. In the mannitol and glucose fermentations it exhibits a continuous rise in potential.

SUMMARY

1. The fermentation of glucose, mannitol, calcium gluconate, and arabinose by the acetone-butyl alcohol microorganism, *Clostridium acetobutylicum*, has been studied with special reference to the relation between the degree of oxidation of the compound fermented and the distribution of the various oxidized and reduced products of fermentation.

2. When mannitol, a reduced compound, is fermented, large

amounts of hydrogen and of butyl alcohol are produced.   The production of acetone is small, and almost as much butyric as acetic acid is formed.   When glucose is fermented, less hydrogen and butyl alcohol and more acetone are produced than are formed from mannitol.   The ratio of acetic to butyric acid is higher in the case of glucose.   Calcium gluconate, which is more oxidized than glucose, is fermented largely into acids, because of the neutralizing effect of the calcium ion, and the oxidized nature of the substrate. Here much more acetone than butyl alcohol is formed, and there is a high acetic to butyric acid ratio.   Hydrogen production, however, being a corollary of acid production, is high.   When arabinose, which has the same degree of oxidation as glucose, is fermented, an "oxidized" type of fermentation is produced. Acetone production is high and butyl alcohol production is low. The acid produced is largely acetic.   The production of hydrogen is small.

3. The appearance of an oxidized type of fermentation from arabinose seems to be related to the ready splitting off of only 1 molecule of carbon dioxide for each molecule of pentose fermented. The resulting scarcity of hydrogen available for reduction results in a large production of a substance whose formation does not involve reduction reactions, namely acetone.   The total amount of carbon dioxide evolved, is, however, more than can be accounted for by a hypothesis predicating the preliminary splitting of the pentose molecule into a two-carbon and a three-carbon compound, and with the subsequent fermentation of these fractions in the conventional manner.

4. The total amounts of carbon dioxide and hydrogen evolved in the fermentations of six-carbon compounds are compatible with the mechanisms which have been proposed for the butyl fermentation by various investigators.   However, the gas production, as well as the balance between the oxidized and reduced compounds produced, indicates the presence in the glucose fermentation of an unknown intermediate product, a precursor of carbon dioxide and hydrogen.

5. A convenient method of drawing up an oxidation-reduction balance for a fermentation is outlined.

6. The oxidation-reduction potentials prevailing in these fermentations have been measured.   Although there is an extremely

rapid fall in potential during the induction period of the fermentation, a hydrogen overvoltage is never developed, except perhaps in the case of the calcium gluconate fermentation.

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A QUANTITATIVE STUDY OF THE GLOMERULAR
ELIMINATION OF UREA IN FROGS

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Philadelphia)*

Quantitative comparison of the composition of glomerular urine
with that of the blood plasma from which it is derived provides
direct evidence as to the nature of glomerular function. A con-
siderable number of such comparisons have already been made in
experiments with the frog and with *Necturus maculosus*. The
majority of these yield the conclusion that glomerular urine is
identical in composition with an ultrafiltrate from the plasma:
that the glomerular process therefore is one of filtration. A small
fraction of the evidence does not uphold this conclusion, but when
the experiments providing this evidence were repeated, their cor-
rectness was not confirmed, and at the same time the possibility of
technical flaws in them was disclosed.

The experiments referred to are in two categories: those in
which comparison was made of total molecular concentration (1,
2) or of total electrolyte concentration (3), and those in which the
concentrations of an individual constituent were compared.
Among the latter are estimations of the concentration of Cl (4, 5)
and of dyes (6) which were in process of elimination during the
glomerular urine collection.

The work here reported constitutes a similar study of urea, the
substance to which possibly greater importance in this connection
attaches than to any other dissolved component of urine. It
seems not unreasonable to assume that if a "secretory" mechanism
is present in the glomerulus for effecting the excretion of any sub-
stance, its existence would be revealed in the elimination of the
most abundant nitrogenous urinary constituent. The outcome,

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however, has not given any indication of the existence in frogs of a glomerular mechanism for "secreting" urea.

The presence of urea in the glomerular urine of frogs was demonstrated in 1925 by Wearn and Richards (7). With the intention of making a quantitative study, they succeeded in so adapting the methods of Benedict and of Folin that amounts of urea of the order of 0.0005 mg. could be determined with satisfactory accuracy (8). The method to be described below possesses the advantages over theirs of applicability to far smaller amounts than this and of far greater ease in performance. Hence the abandonment of their intention in favor of the effort which this paper represents.

The average level of urea nitrogen in the plasma of the frogs which we have used is 5 mg. per 100 cc. With the urea concentration of glomerular urine assumed to be of this order, the urea nitrogen content of 0.5 c.mm. (the volume which can be collected without great difficulty from a single renal corpuscle in 20 to 60 minutes) is 0.000025 mg. Amounts as small as one-tenth of this can be determined by the method which we have used. It consists in mixing an accurately measured sample of glomerular urine with alkaline hypobromite solution in a glass capillary tube and measuring the volume of nitrogen liberated after collecting it in a previously measured air space above the surface of the reaction mixture. This is, of course, a micro adaptation of an old clinical method, long disparaged from the standpoint of accuracy but recently reestablished in repute by the refinements introduced by Stehle (9) and by Van Slyke (10). When working with minute quantities in capillary tubes, it has not been possible to collect the liberated nitrogen *in vacuo* and measure it under standard conditions. Nor is it claimed that any single estimation is more accurate than some 15 per cent. But it will be seen that the plotted results of many estimations of different known solutions group themselves in such a way as to show accurately the concentrations of the solutions used; and further, that when the results of many estimations of urea concentration of glomerular urine are similarly plotted they coincide so closely with the corresponding plasma urea concentrations, determined by the urease method, as to force the conclusion that the urea content of glomerular urine is the same as that of plasma.

Micro Method for Estimation of Urea

1. In Pure Urea Solutions—A supply of capillary tubes was prepared from thoroughly cleaned glass tubing and kept free from dust. Each was about 12 inches long, between 0.2 and 0.5 mm. in diameter, and of uniform diameter throughout its length.

Sodium hypobromite solution was prepared fresh each week by mixing 50 volumes of 40 per cent NaOH with 1 volume of bromine. At least 24 hours before use, a supply was transferred to a small test-tube. Some time before it was used, but not immediately, the gas bubbles which had collected upon the walls of the test-tube were discharged by tapping or revolving the tube.

By means of rubber tubing, attached to one end of a glass capillary tube cut to about 7 cm., five successive columns of glass-distilled water were sucked into it, each separated from its neighbor by a column of air. The fifth column was withdrawn a short distance from the end, and then a column of urea solution from 0.7 to 2.0 mm. long was drawn in. The precise lengths of the alternating columns of water and air are unimportant: those of the column of urea and the adjacent air column are important. They must be short enough to be measured easily with an ocular micrometer. In our experiments a Zeiss ocular micrometer was used, with a lens system such that 1 scale division = 2.4 μ. The columns of urea solution varied between 300 and 900 scale divisions in length. Their measurement must be completed within 15 seconds after the urea solution enters the tube, haste being necessary because of rapidity of evaporation of fluid at the end of a capillary. Within the following minute, the length of the next adjoining column of air was measured.[1] This measurement was repeated until three results agreed within 2 scale divisions. The diameter of the capillary tube was subsequently measured.

The capillary tube was then removed from the microscope stage, the column of urea forced back to its extreme tip, this dipped into the sodium hypobromite, and a volume of reagent from 1 to 6 times that of the urea admitted. After removing the rubber tubing the capillary tube was revolved between thumb and fore-

[1] In this measurement haste is unnecessary. Controls showed that 18 minutes might elapse and the column of urea evaporate to 57 per cent of its volume without affecting the amount of nitrogen evolved.

finger for 10 seconds before it was placed in a nearly perpendicular position upon a plasticine mount. 60 minutes later it was removed from the mount, again revolved for 10 seconds, placed upon the microscope stage, and the air column above the reaction mixture again measured. The increase in length of this column represented, in terms of scale divisions, the amount of nitrogen which had been evolved.[2] It was possible to complete twelve analyses of a urea solution within 2 hours.

2. *In Glomerular Fluid*—In working with glomerular fluid, the procedure was essentially the same. But unless the end of the capillary tube into which the fluid was delivered was dry, the fluid was apt to flow along the wall and mix with the adjacent column of water. On that account, the water columns were introduced from the far end of the capillary and brought down to the near end by inclining the tube or by sucking. When the peripheral water column was at a proper distance from the end, the capillary tube was placed upon the microscope stage, the point of the pipette containing the glomerular fluid inserted into the capillary touching its wall, and a portion or all of the contents of the pipette discharged into the capillary by slightly raising the mercury leveling bulb. The column of glomerular fluid thus formed was measured within 15 seconds, and the subsequent procedures of air measurement and hypobromite admission carried out as has been described. It will be observed that this simple transfer from pipette to capillary was the sole manipulation to which it was necessary to subject the glomerular fluid. If sufficient fluid had been collected for a single estimation only, the transfer was usually completed within 3 minutes after the pipette had been withdrawn from the glomerular capsule. If more than one estimation were to be made on the collected fluid, an interval of about 5 minutes was required for the preparation of each additional capil-

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[2] It was found impracticable to substitute either mercury or oil for the drop of water immediately above the urea; or to seal the lower end of the capillary. It was important that neither the end of the capillary containing the reaction mixture, nor the end of the tube containing the known urea solution, should be agitated or touched with a finger during the manipulation. The only imposed variation in intracapillary pressure occurred when the urea drop was held at the extreme end of the capillary preliminary to admitting the hypobromite. This manipulation was shown to be without effect upon the length of an air column.

lary. Control observations indicated that no evaporation occurred in urea solutions held in the pipette for as long as 26 minutes.

Time of Reading—Measurement of nitrogen evolved from the urea was always made 60 minutes after mixing with the reagent. About 70 per cent of the nitrogen was evolved within the first 10 minutes, but the air column continued to increase in length for 60 minutes. At this point all the evolution of gas which could be attributed to the urea had ceased. During the 2nd and 3rd hours, if observations were continued, there was a slow further increase in the length of the air column amounting to an average of 7.5 scale divisions hourly. This was attributed to an evolution of gas from the sodium hypobromite since control tubes containing only water and hypobromite showed an increase of the same order during these hours. In the 1st hour these control capillaries showed an average increase in the air columns of 8.2 scale divisions (150 observations).

Temperature—The room temperature rarely varied by as much as half a degree during the time that a single set of observations was being made. But from day to day it varied between 21° and 28°. Control experiments showed that the variations in volume of nitrogen caused by even a 7° change in temperature could seldom exceed 2 scale divisions. There was no demonstrable relationship between temperature changes and the variations in our results. Therefore no correction for these changes was applied and no attempt was made to work at a more constant temperature.

Size of Capillary Tube—The size of the tube chosen (within the range of 0.2 to 0.5 mm. inner diameter) depended solely upon the amount of fluid available. Granted a column of reasonable length (more than 300 scale divisions), the method is quite as accurate with a small column of fluid in a small capillary tube (0.05 c.mm.) as with a large column in a large tube (0.43 c.mm.). The gas is liberated more rapidly in a large capillary, but even in a small capillary this is completed well within 60 minutes. Capillaries smaller than 0.2 mm. are inconvenient to handle. If the concentration of the fluid is much above 25 mg. of urea nitrogen per 100 cc., a 0.2 mm. capillary is too small, for the nitrogen bubbles will not break spontaneously. The average diameter of the capillaries employed was 0.34 mm.

Amount of Reagent—The amount of sodium hypobromite used

varied from 1 to 6 (usually 2) times the volume of the urea solution. From such variations no difference in the results could be detected. When the dilution was more than 6 the yield of nitrogen was often diminished below the average: presumably because of the solvent effect of the excessive amount of fluid.

Effect of Protein—The presence of protein in the fluid to be tested causes the yield of nitrogen to be greatly increased. Attempted estimations on three specimens of bladder urine and one glomerular urine (from a frog which had received a previous injection of mercury bichloride) were abandoned on this account.

Non-Urea Nitrogen Liberated by Reagent—Sodium hypobromite is not a specific reagent for urea. It effects the complete release of nitrogen from ammonia. We did not attempt to make either glomerular urine or plasma ammonia-free. Our measurements of "urea nitrogen" therefore include such minute amounts of ammonia nitrogen as may have been present in either fluid. The reagent also liberates approximately 50 per cent of the available nitrogen from uric acid and creatinine. If these substances are present in glomerular fluid in the same concentrations in which they exist in plasma, their presence would add to the apparent urea concentration of the fluid. Macro analyses of frog plasma however indicate an average uric acid content of less than 0.5 mg. per 100 cc., an average total creatinine content of 0.9 mg. per 100 cc.; and analyses of uric acid and creatinine by the hypobromite method show that such concentrations would not increase the apparent urea nitrogen concentration of glomerular fluid by more than 0.2 mg. per 100 cc. Of the five amino acids which we have investigated (arginine, cysteine, glycocoll, leucine, and tyrosine) only one, arginine, yields nitrogen with sodium hypobromite. We have made, in triplicate, eleven hypobromite analyses of five specimens of frog plasma which were also analyzed by the macro urease titration method. In no instance did the urea content developed by the micro method exceed that developed by the macro method by more than 10 per cent, and the average difference was only 0.3 per cent. It is apparent therefore that the liberation of non-urea nitrogen by sodium hypobromite from frog plasma (and, presumably, glomerular urine) is insufficient to introduce any recognizable error into our determinations. We have therefore made no correction for this error

which, if it were present, would have the effect of making the glomerular urine appear to be hypertonic to plasma in its urea content.

Accuracy of Hypobromite Determinations—On each day of the 2 months on which glomerular fluid was collected and studied by this method, three or more estimations were made by the same method upon similar amounts of urea solutions of known strength. These control results were plotted on a graph in which abscissæ represented nitrogen evolved, ordinates the amount of urea used in the determination.

Chart 1 shows 177 such estimations. The open circles represent group averages, and the line[3] drawn through them represents the average volume of nitrogen, expressed in scale divisions, which is evolved from any amount of urea within the range of the graph (0.000001 to 0.000070 mg. urea nitrogen). When the diameter of the capillary tube is taken into account and the nitrogen computed in c.mm. these averages are found to constitute 93.7 per cent of the calculated nitrogen present. If the line were corrected (1) for this deficit in our method (6.3 per cent) and (2) for the average volume of gas liberated from reagents alone (8.2 scale divisions) it would correspond precisely with the amount of nitrogen theoretically yielded by any amount of urea within the range of our graph.

When the amount of nitrogen evolved in each single estimation of this series is interpolated on this line, and from this the concentration of each solution is calculated, it is found that 51 per cent (90) of the 177 observations are within 10 per cent of the true value as determined by titration; 73 per cent are within 15 per cent; only 5 per cent differ by more than 25 per cent from the true value. The great majority of the aberrant results were obtained with the two weakest solutions, both of these containing less than 5 mg. per 100 cc.

The accuracy of the method was distinctly improved when we relied on the average of a group of readings rather than on the result of a single estimation. It was our practice to make estimations in groups of three; and, in Table I, we have listed the averages

[3] This line reaches the abscissa at 8 scale divisions because some gas is evolved from sodium hypobromite and water in the absence of urea (see p. 597).

of these groups in comparison with the known value of the solution. Of the 57 groups, 74 per cent show averages within 10 per cent and 88 per cent within 15 per cent of the known value. Again the accuracy was least with the weakest solutions; when solutions
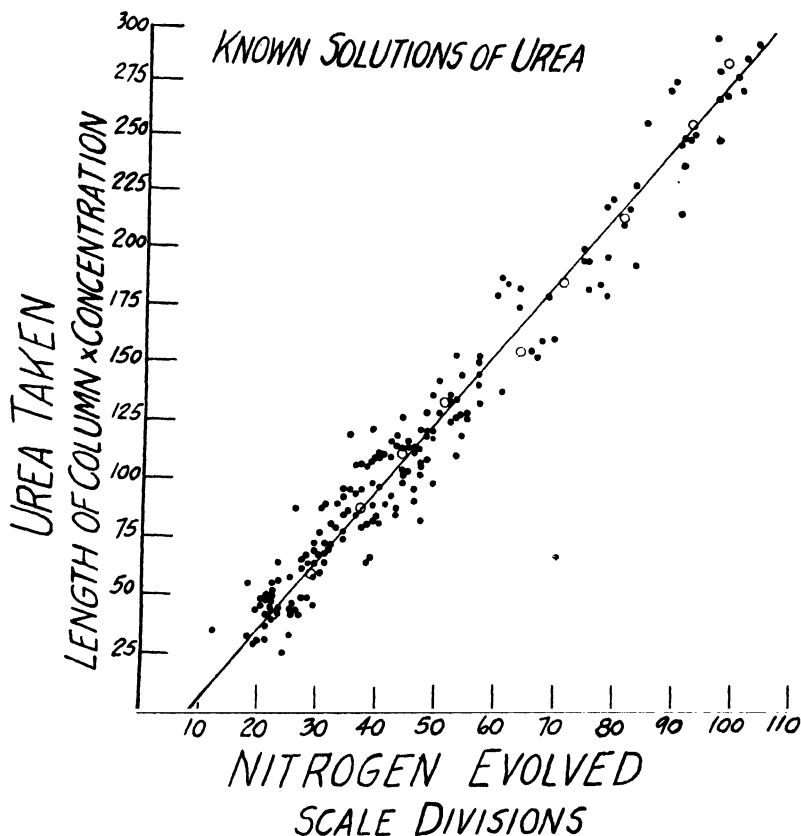
CHART 1. Results of 177 determinations on known urea solutions by the capillary hypobromite method. The ordinates show amounts of urea taken, expressed as length of column of urea solution in scale divisions × concentration of the solution in decigrams of urea per 100 cc. The abscissæ show amounts of nitrogen evolved, expressed as scale divisions. Linear measurements only are presented since, in each determination, the diameter of the column of urea solution was the same as that of the air space in which the nitrogen was collected and measured. Open circles represent group averages and the line is drawn to represent the curve of these.

TABLE I

Determinations in Triplicate on Known Solutions by the Capillary Hypo-bromite Method

| Date | Urea N per 100 cc. | | Error |
	Known solution	Average of three determinations	
1930	*mg.*	*mg.*	*per cent*
Apr. 19	23.4	24.2	+3.4
May 24	23.4	22.9	−2.1
			+0.7 Mean
			2.8 " deviation
Apr. 11	17.1	18.1	+5.9
" 14	17.1	16.0	−6.4
" 15	17.1	16.5 (5)*	−3.5
" 19	17.1	18.1	+5.9
" 22	17.1	17.6	+2.9
" 28	17.1	18.0	+5.3
			+1.7 Mean
			4.4 " deviation
May 14	12.6	11.7 (2)	−7.1
" 19	12.6	13.3	+5.6
" 20	12.6	12.0	−4.8
" 21	12.6	11.4	−9.5
" 22	12.6	13.1	+4.0
" 23	12.6	12.9	+2.4
" 27	12.6	13.0 (2)	+3.2
" 28	12.6	12.8	+1.6
" 31	12.6	12.0	−4.8
June 5	12.6	12.7 (5)	+0.8
			−0.9 Mean
			4.6 " deviation
Mar 31	7.5	6.6	−12.0
" 31	7.5	7.0 (4)	−6.7
Apr. 1	7.5	7.2	−4.0
" 3	7.5	6.9	−8.0
" 4	7.5	7.2	−4.0
" 5	7.5	7.2	−4.0
" 7	7.5	7.6	+1.3
" 9	7.5	8.1	+8.0
" 9	7.5	7.6	+1.3
" 10	7.5	8.1	+8.0
" 10	7.5	6.7	−10.7
" 12	7.5	7.4	−1.3
" 17	7.5	6.6	−12.0
" 17	7.5	8.1	+8.0

* Denotes number of estimations when other than three.

TABLE I—*Concluded*

| Date | Urea N per 100 cc. | | Error |
	Known solution	Average of three determinations	
1930	*mg.*	*mg.*	*per cent*
Apr. 19	7.5	7.7	+2.7
" 23	7.5	7.4	−1.3
" 24	7.5	6.5	−13.3
" 29	7.5	7.0	−6.6
" 30	7.5	7.0 (2)	−6.6
May 1	7.5	7.0 (2)	−6.6
" 2	7.5	6.7	−10.7
" 3	7.5	8.0	+6.6
" 12	7.5	6.9 (2)	−8.0
" 13	7.5	6.6	−12.0
" 22	7.5	7.5 (2)	0.0
			−3.7 Mean
			5.5 " deviation
May 13	4.9	5.5	+12.2
" 14	4.9	3.8	−22.4
" 19	4.9	5.0 (2)	+2.0
" 20	4.9	6.0	+22.4
" 21	4.9	4.9	0.0
" 26	4.9	4.3	−12.3
" 27	4.9	5.8 (2)	+18.4
" 29	4.9	5.2	+6.1
			+3.3 Mean
			11.5 " deviation
Apr. 17	3.8	4.1 (2)	+7.9
" 19	3.8	3.5 (4)	−7.9
June 3	2.45	3.6 (2)	+46.9
" 3	2.45	3.0	+22.4
" 3	2.45	2.8	+14.3
" 3	2.45	2.0	−19.6
			+10.7 Mean
			17.2 " deviation
Mean....................................			+0.1

stronger than 5 mg. per 100 cc. were used, no one of the groups showed an error of as much as 15 per cent.

From these results, we conclude that the error of a single estimation is relatively large; but that when estimations are made in triplicate the error of the average is usually less than 10 per cent.

And when averages of a large series of estimations are taken the resulting accuracy is quite comparable with that of any of the more refined methods now current for the estimation of urea nitrogen.

TABLE II

Comparisons of Urea Determinations on Bladder Urine by the Capillary Hypobromite and Urease Titration Methods

| Date | Urea N per 100 cc. | | Difference |
	Hypobromite method (average of three determinations)	Urease titration method	
	Diluted human urine		
1930	*mg.*	*mg.*	*per cent*
Feb. 1	24.0(1)*	23.8	+0.8
" 3	26.5	23.4	+13.2
" 5	22.8	23.9	−4.6
" 5	29.6(2)	26.9	+10.0
" 5	28.3	28.0	+1.1
	Frog bladder urine		
Mar. 7	27.6	26.3	+4.9
" 7	25.8(2)	24.4	+5.7
" 7	25.0	24.4	+2.5
" 13	17.1	14.9	+14.8
" 15	27.2(2)	27.3	−0.4
" 18	24.0	25.7	−6.6
" 19	33.7(2)	36.6	−7.9
" 19	22.8	25.7	−11.3
" 19	19.3(2)	16.0	+20.6
" 21	16.8	16.0	+5.0
" 21	36.8	37.0	−0.5
" 21	26.1	25.7	+1.6
" 21	27.4	29.6	−7.4
" 22	18.6	21.2	−12.3
" 24.	22.0(1)	21.2	+3.8
Mean of series on fifteen frogs......		24.8	+0.8

* Denotes number of estimations when other than three.

Bladder Urine—Before attempting to apply the method to glomerular urine, a series of twenty estimations was made upon bladder urines. Fifteen of these were obtained from normal frogs;

five were human urine, properly diluted. A series of (usually) three hypobromite determinations was made on each specimen, and the average of these determinations compared with a single macro determination made by the urease titration method. This comparison is presented in Table II. Fifteen of the comparisons (75 per cent) agree within 10 per cent, and in only one instance is the disagreement greater than 15 per cent.

Estimations of Plasma Urea

We repeatedly attempted to deproteinize without dilution the small amounts of plasma that could be obtained from the frog during our glomerular collection, so that estimations might be made on specimens obtained at this time and by the capillary hypobromite method. These attempts were unsuccessful.[4] Accordingly the macro urease titration method of Van Slyke and Cullen (11) was employed. Justification for comparing the results of analyses made by the two methods lies in the results of our estimations on known solutions of urea and bladder urine, and in the close agreement obtained in comparisons of the macro hypobromite and urease titration methods by Stehle and Van Slyke on bladder urine.

Immediately after the pipette was withdrawn from the glomerulus the frog was bled from the aorta. The average volume of plasma obtained was 0.65 cc. Heparin was used as anticoagulant. One blank determination and two determinations on known solutions of urea (the same as those used in control of the hypobromite method) were made with each specimen of plasma so that appropriate correction might be made to the plasma reading. This correction averaged 3 per cent and exceeded 6 per cent in only three instances. No great reliance is placed on the accuracy of the method with plasmas containing less than 5 mg. of urea nitrogen per 100 cc.; an error of a single drop in the titration at these levels may make a difference of as much as 30 per cent in the calculated value; and six out of twenty-eight determinations on known urea solutions of this strength gave errors of from 9 to 15 per cent.

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[4] Subsequent to the completion of the experiments described in this paper we found the capillary hypobromite method applicable to amounts of frog plasma of the order of 0.1 cc. with an accuracy comparable to that observed in the case of urea solutions and bladder urine.

Plasma, because of its protein content, contains approximately 3 per cent less water than does glomerular urine. We have therefore added a 3 per cent correction to the values obtained by titration.

Two objections may properly be raised against a comparison between glomerular urine, and plasma obtained in the manner described. The ideal time to obtain the plasma specimen is in the

TABLE III

Effect of Intravenous Saline upon Urea Content of Frog Plasma*

Urea N per 100 cc.		
Before saline	After saline	
mg.	*mg.*	*per cent*
2.6	2.5	−3.8
3.7	3.8	+2.7
3.7	3.6	−2.7
3.9	3.8	−2.5
4.2	4.6	+9.5
4.3	4.6	+6.9
4.6	4.2	−8.7
4.8	4.1	−14.6
4.8	4.4	−8.3
4.9	4.8	−2.0
5.2	5.8	+11.5
5.4	6.4	+18.5
6.0	4.7	−21.6
6.1	6.0	−1.6
17.6	16.6	−5.7
33.0	33.2	+0.6
Mean...		−1.4

* In each experiment the first blood sample was taken immediately before, the second 10 to 15 minutes after, the intravenous injection of 0.5 to 1.0 cc. of 0.6 per cent sodium chloride solution.

precise middle of the glomerular collection. This was not done. There was an average interval of 42 minutes between the middle of the glomerular collection and the end of the plasma collection. To investigate the effect of this delay, at intervals of from 30 to 60 minutes two samples of plasma were taken from each of thirteen frogs. In no instance was the difference in urea content between these specimens greater than the experimental error of the method.

A second objectionable point in our procedure is the fact that, in twenty of the forty-three experiments, though the renal circulation had continued active, the heart was adjudged insufficiently vigorous to yield as much as 0.5 cc. of plasma. From 0.5 to 1.0 cc. of 0.6 per cent sodium chloride solution was therefore injected intravenously and, after an interval of 10 to 15 minutes, bleeding from the aorta was commenced. A series of sixteen control observations summarized in Table III indicates that this procedure has surprisingly little effect upon the plasma level of urea. In only four instances is the change greater than 10 per cent and the average difference is minus 1.4 per cent. This injection may introduce an error into any single experiment, but the error is rarely a large one and disappears in a considerable series. Nor does examination of our results detect any systematic difference between the experiments in which saline was injected and those in which its injection was unnecessary. We do not therefore exclude these twenty experiments.

Details of Experiments

The forty-three experiments outlined in Table IV were made between March 31 and May 31 on forty healthy frogs (*Rana pipiens*). The animals varied in weight from 29 to 75 gm., and all but five were females. 3 cc. of 0.6 per cent sodium chloride solution were injected subcutaneously at the time the brain was destroyed by hemostat. No more saline was injected save in the single instance mentioned in Table IV. The right kidney was exposed for observation in the usual manner. The collection of glomerular urine was commonly started within 35 minutes after beginning the dissection, and lasted for an average of 34 minutes; in only four instances did the collection continue for more than 45 minutes, a deliberate effort being made to keep the collection time brief because of the impossibility of obtaining plasma samples before the collection was finished. The circulation within the tuft from which collection was being made was always good throughout the entire period of collection except in the four instances to which allusion is made in Table IV. The amount of glomerular fluid collected varied between 0.04 and 0.65 c.mm. Except in three instances the mercury leveling bulb connected with the pipette was either at the kidney level or from 1 to 8 mm. above it. As an addi-

tional safeguard against contamination of the glomerular collection with tubular fluid, an attempt was made in every instance to obstruct the tubule leading from the punctured glomerulus by forcefully compressing it with a blunt glass rod. In eleven experiments the obstructing rod was simply brought down at a point in relation to the glomerulus at which experience has taught us the connected tubule was most likely to lie. In thirty-two experiments a preliminary intracapsular injection of about 0.5 mg. of 0.15 per cent phenol red was made in order more accurately to localize the tubule.[5]   In Table IV these two methods are respectively indicated as "guess" and "phenol red." Usually the injection of phenol red allows one to visualize the tubule with precision from the point where it leaves the capsule, and to impose the block with accuracy and close to the glomerulus. Occasionally the tubule leaves the capsule in a dorsal direction and only comes to the surface at some distance from the glomerulus. Under these circumstances, designated in Table IV as "not precise" the obstructing rod is upon a portion of the proximal tubule rather than upon the tubule neck. Throughout each collection we repeatedly assured ourselves of the intracapsular position of our pipette point by momentarily raising the mercury bulb and observing the consequent changes in the size of the capillary tuft.

When our first experiments revealed the very low level of plasma urea, repeated efforts were made to increase it by the administration of urea in order to lessen the relative importance of analytical error and to encourage such secretory activity as might exist. These efforts were largely unsuccessful. Some of the frogs were placed for 24 to 48 hours in water containing 20 to 40 mg. per cent of urea; others received from 2 to 50 mg. of urea by mouth, or subcutaneously, at varying periods before the experiment. In each series the average level of urea was somewhat higher than in the uninjected frogs, but in only two instances was it beyond figures which had been previously encountered. In a single experiment the plasma urea was raised by the previous injection of 0.3 mg. of mercury bichloride.

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[5] In three instances, noted in Table IV as "tubule not blocked," this identification was unsuccessful.

TABLE IV

Urea Content of Glomerular Urine and Blood Plasma from Frogs

1. Experiments in which plasma contained more than 7.5 mg. of urea N per 100 cc.

Experiment No.	Method of identifying tubule	Glomerular collection			Data of determinations on glomerular urine			Urea N per 100 cc. in		Difference	Notes
		Pressure	Volume	Time	Glomerular urine	N evolved	Concentration of urea N	Glomerular urine	Plasma		
		mm. Hg	*c. mm.*	*min.*	*scale divisions*	*scale divisions*	*mg. per 100 cc.*	*mg.*	*mg.*	*per cent*	
5†	Guess	+3	0.26	33	930	96	12.8	12.80	11.50	+11.3	0.3 cc. intravenous saline during collection
6	"	+3	0.33	27	940	61	7.9	7.90	8.41	−6.1	7.9 may have been 8.4
23††	Phenol red	+2	0.20	35	850	68	9.9	9.90	13.20	−25.0	
28††	" " , not precise	+2	0.04	38	220	24	10.2	10.20	11.34	−10.0	Circulation sluggish. 10.2 determination on short column
32†	Phenol red	+2	0.08	21	550	66	14.7	14.70	17.50	−16.0	Circulation sluggish. Glomerular fluid contaminated (about 5 per cent) with urea-free fluid
35†	" "	+3, −7; −20, −32	0.40	35	440; 420; 375	104; 89; 85	29.0; 27.0; 28.6	28.20	26.60	+6.0	Tubule block very accurate
36†	" " , not precise	?	0.10	29	540	60	13.4	13.40	14.25	−6.0	

38‡	Phenol red	+3	0.37	40	400 420 700	41 36 62	10.8 8.8 10.8	10.13	9.88	+2.5	Circulation sluggish. Frog received 0.3 mg. HgCl₂ 5 days previously
40‡	" "	0	0.60	26	630	44	7.9	7.90	8.04	−1.7	
44†	" "	+2	0.23	73	590 460	82 60	17.6 18.5	18.05	18.05	0.0	
51‡‡	" "	+5	0.18	40	580	43	8.4	8.40	8.70	−3.5	Plasma collection delayed 45 min.
53‡‡	" ", not precise	+4, +1	0.15	34	660	72	13.5	13.50	11.62	+16.2	
54‡‡	Phenol red	+4	0.14	17	600	49	9.5	9.50	8.33	+14.1	Circulation sluggish
										−1.4 Mean	
										9.0 " deviation	

2. Experiments in which plasma contained more than 4.9 but less than 7.5 mg. of urea N per 100 cc.

10	Guess	+3	0.35	36	700 550	35 27	5.3 4.7	5.00	5.34	−6.4	One urine determination omitted
17‡‡	Phenol red	+3, +1	0.14	43	900 180	65 19	8.8 8.3	8.55	7.30	+17.1	8.3 determination on short column
22‡	Guess	+2	0.14	43	770	35	4.9	4.90	6.17	−20.6	Glomerular capsule torn
27‡	Phenol red, not precise	+2	0.43	19	600 250	34 17	5.9 4.7	5.30	6.03	−12.1	Same frog as Experiment 26, different glomerulus. Circulation excellent. 5.9 more reliable than 4.7
37‡	Phenol red	+5	0.10	22	560	31	5.7	5.70	6.88	−17.2	
42‡	Guess	+3	0.39	21	960	60	7.6	7.60	6.89	+10.3	
43‡	Phenol red, not precise	+3	0.13	10	270	21	6.5	6.50	6.89	−5.2	Same frog as Experiment 42, different glomerulus

* The recorded volume is often approximate, a portion of the fluid being lost during its transfer.

† In these experiments saline was injected intravenously before plasma was collected.

‡ Preliminary to Experiments 15 and 36 to 43, the frogs were placed in urea solution. Urea was administered by mouth in Experiments 17 to 24 and subcutaneously in Experiments 26 to 29, 35, and 50 to 54.

TABLE IV—Continued

Experiment No.	Method of identifying tubule	Glomerular collection			Data of determinations on glomerular urine			Urea N per 100 cc. in		Difference	Notes
		Pressure	Volume	Time	Glomerular urine column	N evolved	Concentration of urea N	Glomerular urine	Plasma		
		mm. Hg	c. mm.	min.	scale divisions	scale divisions	mg. per 100 cc.	mg.	mg.	per cent	

2. Experiments in which plasma contained more than 4.9 but less than 7.5 mg. of urea N per 100 cc.—*Concluded*

Experiment No.	Method of identifying tubule	Pressure	Volume	Time	Glomerular urine column	N evolved	Concentration of urea N	Glomerular urine	Plasma	Difference	Notes
46	Tubule not blocked	+8, 0	0.29	18	1000	47	5 4	5.40	5.56	−2.9	
48	Phenol red	+1	0.32	23	615 / 760	34 / 34	5.8 / 4.8	5.30	5.30	0.0	Same frog as Experiment 47, different glomerulus. Circulation excellent. 5.8 may have been 5.5
50††	" "	+5	0.23	40	480	32	6.9	6.90	7.25	−4.8	One urine determination omitted
52††	" "	+4, +1	0.46	55	750 / 650	37 / 27	5.3 / 4.0	4.65	5.05	−7.9	5.3 may have been 6.3
2	Guess	−2	0.13	25	435 / 400	21 / 19	4.0 / 3.7	3.85	6.78	−43.2	
47	Phenol red	+1	0.65	31	560 / 770 / 870 / 420	41 / 54 / 57 / 35	8.1 / 8.3 / 7.9 / 8.8	8.28	5.30	+56.0	Same frog as Experiment 48. Plasma collection delayed 62 min. Point first entered tubule beneath tuft

−2.8 Mean
14.6 " deviation

3. Experiments in which plasma contained less than 4.9 mg. of urea N per 100 cc.

											Remarks
3	Guess	+2	0.49	30	400 390 710	16 21 29	2.7 4.6 4.1	3.80	3.46	+9.8	4.1 determination unreliable
4	"	+2	0.33	38	650 1100	24 33	3.4 3.1	3.25	4.58	−29.0	
7	"	+3	0.40	44	570 750 710	19 26 17	2.6 3.3 1.7	2.53	2.20	+15.0	Bladder urine, 20.2 mg. urea N per 100 cc.
13†	Phenol red	+8, +5, +3	0.45	43	650 700	26 23	3.8 2.9	3.35	3.10	+8.1	
15††	"	+3	0.41	30	560 610	26 25	4.4 3.8	4.10	3.42	+19.9	4.4 may have evaporated
21††	"	+3	0.08	44	450	20	3.7	3.70	4.57	−19.0	
24†	" , not precise	+2	0.10	21	260	18	4.6	4.60	4.60	0.0	4.6 may have been 5.1
29††	Guess	+2	0.08	24	260	12	2.1	2.10	2.15	−2.3	2.1 determination on short column
30	Phenol red	+2	0.20	30	530	21	3.3	3.30	3.83	−13.8	
31	"	+2	0.16	23	875	36	4.4	4.40	3.83	+14.9	Same frog as Experiment 30, different glomerulus
33†	" , not precise	+2	0.09	38	575	27	4.6	4.60	4.40	+4.5	
34	Phenol red	+3	0.24	33	575 550	28 33	5.0 6.3	5.65	4.35	+29.9	
39††	"	+3, 0	0.25	55	740 470	29 19	3.9 3.2	3.55	3.83	−7.3	
41††	"	+3, 0, −7	0.30	41	540 620 510	20 27 20	3.0 4.2 3.2	3.47	3.50	−0.9	Tubule block very accurate

+2.1 Mean
12.5 " deviation

TABLE IV—*Concluded*

Experiment No.	Method of identifying tubule	Glomerular collection			Data of determinations on glomerular urine			Urea N per 100 cc. in		Difference	Notes
		Pressure	Volume	Time	Glomerular urine column	N evolved	Concentration of urea N	Glomerular urine	Plasma		
		mm. Hg	*c. mm.**	*min.*	*scale divisions*	*scale divisions*	*mg. per 100 cc.*	*mg.*	*mg.*	*per cent*	
9†	Guess	+3, 0	0.40	73	560 600 910 690	94 104 115 97	20.9 21.8 16.2 17.7	19.15	8.98	+113.2	Same frog as Experiment 27. Plasma collection delayed 64 min. Bladder urine contained large amount of urea
26†	Tubule not blocked	+2	0.52	15	700 800	55 52	9.4 7.6	8.50	6.03	+40.9	
49†	" "	+4, +1	0.15	35	520	30	5.7	5.70	4.07	+40.0	
Mean..........		+2	0.27	34							

4. Experiments to which special reference is made on p. 615

Excluding Experiment 9.... +1.3

<center><i>Results</i></center>

The details of forty-three experiments in which the urea concentrations of glomerular urine and plasma have been determined are summarized in Table IV. Excluding Experiment 9 (see below) the average value of glomerular urine urea is 1.3 per cent higher than that of plasma urea, a difference so far within experimental error as to be insignificant. This average is made up from twenty-two results in which glomerular urea is higher, and twenty results in which it is equal to or lower than the plasma urea. In twenty-eight instances the difference was less than 15 per cent.

These comparisons are shown graphically in Chart 2. In constructing it, the curve of averages of 177 control micro estimations on known urea solutions has been used as the curve of plasma urea values against which the glomerular urea figures have been plotted. That is to say, the urea nitrogen concentration of any single plasma specimen having been estimated by the urease titration method, interpolation on this line showed how much nitrogen might be expected to be evolved in the micro estimation from the sample of glomerular urine, on the assumption that this has the same concentration as that of the plasma. The volume of nitrogen actually evolved was then charted at the appropriate point to the right or left. Hence the dispersion of these points with reference to the curve of plasma values is strictly comparable with the dispersion of the points in Chart 1 with reference to the curve of averages. In nineteen of the forty-two experiments sufficient glomerular fluid was collected to make possible two or more analyses; all of the 68 determinations are included in Chart 2.

It is at once apparent that the sum of these comparisons of glomerular urine urea and plasma urea is similar in character to that of the comparisons between known and found values of pure urea solutions. The dispersion is somewhat greater, but it is to be remembered that the curve of plasma urea values is constructed from single estimations, often on amounts as small as 0.5 cc.; and further, that in the majority of cases the urea content of the plasma and of the glomerular urine was so low as to accentuate the effects of errors of analytical technique in both methods.

In Table IV the experiments have been divided into three groups according to the urea nitrogen content of the plasma. In no one of these groups is the average difference between glomerular urine
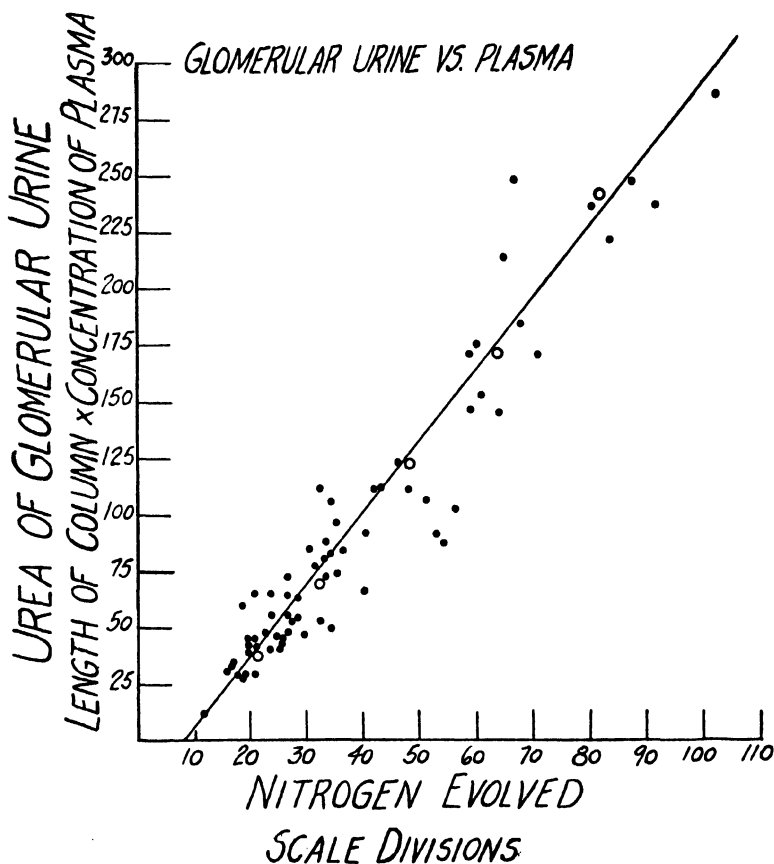
CHART 2. Results of 68 comparisons between the urea nitrogen contents of glomerular urine and plasma. Open circles represent group averages. The line in this chart is in the same position as that of Chart 1. In placing the points on the ordinates, the length of the glomerular fluid column (in scale divisions) was multiplied by the concentration of the plasma (in decigrams of urea per 100 cc.) as determined by urease titration. The horizontal displacement of these points from the curve therefore indicates the difference by which the actual yield of nitrogen differed from the nitrogen theoretically yielded by a fluid with the precise urea content of the plasma.

and plasma more than 3 per cent; but in the individual experiments the difference becomes greater as the urea concentration decreases. This parallels our experience with known solutions of similar strengths; where the percentage error of individual determinations becomes progressively greater as the concentration of urea is reduced. The column of notes in Table IV includes information useful in evaluating the individual results. The last three experiments in Table IV are those in which the wide divergence of values between plasma and glomerular urine urea seemed explicable on technical grounds. In Experiment 9 it was highly probable that glomerular urine was contaminated with fluid from the tubule. In Experiment 26, failure to block the tubule may have been the cause of the apparently high glomerular urea value. Another collection in the same frog from another glomerulus, the tubule of which was satisfactorily blocked (Experiment 27), showed essential identity with plasma. In Experiment 49, the urea concentration was low and the tubule was not blocked. A total of seventeen experiments has been excluded from Table IV and thus far from this account. In six of these, the duplicate determinations made upon each collection of glomerular urine were so discordant as to make an average meaningless. We have no explanation to offer. On the same days on which these experiments were made, precisely similar discordances were encountered in the micro analyses of known urea solutions. In view of the minuteness of the quantities of urea with which we have been concerned, it is remarkable that such discordances were not more frequent. Four experiments[6] were excluded because of loss of the plasma urea estimations. In one experiment the urea nitrogen concentration was so low (0.61 mg. per 100 cc. of plasma) as to make impossible its accurate analysis in either fluid. The other experiments not included were discarded either because the volume of glomerular fluid collected was too small for satisfactory analysis, because of known contamination with surface fluid, or because of the presence of protein in the glomerular urine.

[6] Urea nitrogen values of glomerular urine in these were 4.0, 4.6, 8.9, and 11.4 (average 7.1) mg. per 100 cc.

SUMMARY AND CONCLUSIONS

A technique has been developed for utilizing the urea-hypo-bromite reaction in quantitative estimations of the urea content of the glomerular urine from frogs. In control experiments the method was found to be applicable to amounts of urea nitrogen as small as 0.000003 mg., in volumes of fluid as small as 0.05 c.mm. The probable accuracy of a single estimation is not great; that of the average of a series of estimations is surprisingly high. Applied to known solutions, the mean recovery was 94 per cent of the calculated nitrogen.

Forty-three successful experiments have been made in which the urea content of glomerular urine, determined in 72 analyses by the micro hypobromite method, has been compared with the plasma urea content determined by the urease titration method. The results indicate identity of urea concentration in the two fluids, and further support the view that the glomerular function is one of filtration.

The difference between the concentration of urea in frog plasma and that in the bladder urine (about 5-fold) is readily explainable on the basis of what is already known concerning reabsorption of water from the renal tubule. In the light of the data here supplied, it is not necessary to assume tubular secretion of urea to account for the renal elimination of this substance in the frog.

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STROPHANTHIN

XX. THE CONVERSION OF ISOSTROPHANTHIDIC ACID
INTO THE DESOXO DERIVATIVE

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The cardiac aglucones, the formulations of which have now been
definitely established, are as follows:

Strophanthidin...................................... $C_{23}H_{32}O_6$
Digitoxigenin...................................... $C_{23}H_{34}O_4$
Gitoxigenin...................................... $C_{23}H_{34}O_5$
Digoxigenin[1]...................................... $C_{23}H_{34}O_5$
Periplogenin...................................... $C_{23}H_{34}O_5$
Sarmentogenin...................................... $C_{23}H_{34}O_5$

The close structural relationship of these aglucones, which is at
once superficially suggested by these formulations, has been more
definitely emphasized by common features in structure which have
come to light during their individual investigation. They have
all been found to be polyhydroxytetracyclic-$\Delta^{\beta,\gamma}$-lactones which
under the influence of alkali may be isomerized to saturated sub-
stances. The extent, however, to which these structural resem-
blances would be maintained remained a subject for further inquiry
and one of great importance in the problem. If the individual
aglucones could be separately correlated with strophanthidin, the
structural investigation of the group would then be greatly
simplified.

Strophanthidin differs from the other aglucones (with the excep-
tion of antiarigenin) by its possession of an aldehydic carbonyl

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[1] Smith, S., *J. Chem. Soc.*, 509 (1930). Mannich, C., Mohns, P., and
Mauss, W., *Arch. Pharm.*, **268**, 453 (1930).

group. By the conversion of strophanthidin into a desoxo derivative a substance of the formula $C_{23}H_{34}O_5$ would result, which might be directly compared with the above naturally occurring aglucones of the same formulation. For purposes of correlation, therefore, the transformation of this aldehyde group to methyl has been made the subject of careful study by us.

Strophanthidin itself was, however, found to be useless for this purpose because of its general susceptibility to most reagents, with the formation of obscure transformation products. We have turned, therefore, to certain derivatives of strophanthidin which appeared to be more suitable, namely isostrophanthidic acid[2] and the hydrogenated anhydrostrophanthidins. Our experience with the latter will be presented in a separate communication.

In the case of isostrophanthidic acid, it was found possible to accomplish the desired transformation into the desoxo derivative by the use of the method of Wolff, after the miscarriage of other methods. After heating the *semicarbazone* with sodium ethylate, a crystalline acid could be separated from the reaction mixture, although in poor yield; this was definitely shown to possess the formula $C_{23}H_{34}O_6$. This was confirmed by the analysis of its *methyl ester* and, as will be presented in the following paper, by its identification with *isoperiplogenic acid*.

Previous to the successful use of the method of Wolff we had attempted to apply the method of Clemmensen. When this was carried out in acetic acid solution by the usual procedure, only non-crystalline reaction products were obtained. When, however, methyl alcohol was used as the solvent, simultaneous esterification occurred and it was possible to obtain in rather poor yield a neutral crystalline substance. The analysis of this reaction product gave results which agreed with the formulation $C_{24}H_{36}O_7$. Investigation showed that the aldehyde group had been reduced to the primary alcoholic group.[3] Simultaneously, isomerization

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[2] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **61**, 387 (1924).

[3] The reduction of the aldehyde group to the CH_2OH group has already been described in the case of the formation of dihydrostrophanthidol (Jacobs, W. A., *J. Biol. Chem.*, **88**, 528 (1930)). Our experience with the above aglucones has shown no evidence of the presence in them of such a primary alcoholic group although it is probable that such aglucones may be found in nature.

to the γ⁴ series occurred under the influence of the conditions used, since the substance (γ-isostrophanthidolic methyl ester) was strongly dextrorotatory. When a modified Clemmensen reaction was performed at lower temperature, the resulting substance gave similar analytical figures but like α-isostrophanthidic acid and its ester proved to be weakly levorotatory ($[\alpha]_D$ = −14.0) and was therefore *α-isostrophanthidolic methyl ester.*[5] On a previous occasion we have already noted the fact that α-isostrophanthidic acid itself, at least under the conditions used, apparently is not converted into a γ-isomer. This is very curious in view of the fact that the corresponding acid, α-isostrophanthic acid, and the primary alcohol now described undergo such isomerization.

The effort was made to replace the primary hydroxyl group of this reduction product by halogen with the hope of subsequent reduction of the resulting CH_2X group to methyl. But the usual reagents such as the halogen acids, the phosphorus halides, and thionyl chloride gave us no tangible result. In order to protect the possibly interfering hydroxyl groups already present in isostrophanthidic methyl ester, an attempt was made to acetylate them. By the direct use of acetyl chloride, however, only the secondary hydroxyl (OH''') could be directly acylated with the formation of *acetyl-α-isostrophanthidic methyl ester.* Further studies were therefore made with this derivative. On catalytic reduction its aldehyde group was reduced with difficulty to the primary alcoholic group with the formation of *acetyl-α-isostrophanthidolic methyl ester.* Attempts to replace the primary hydroxyl group of this substance by halogen were unsuccessful. When it was treated with thionyl chloride, the acetyl group was replaced by thionyl with the formation of a neutral sulfite in which the primary and secondary hydroxyl groups were bridged by SO. In the course of these studies it was found that isostrophanthidic methyl ester also forms a neutral sulfite when treated with thionyl chloride; but in this case the SO group bridged the tertiary hydroxyl (OH'') and the secondary hydroxyl (OH''').

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[4] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 831 (1927).

[5] Reduction to the primary alcohol was also accomplished by the use of sodium amalgam after saponification of the lactone group of α-isostrophanthidic acid and neutralization with acetic acid and addition of $(NH_4)_2$-SO_4 as a buffer.

Semicarbazone of α-Isostrophanthidic Acid—To a solution of 10 gm. of α-isostrophanthidic acid in 800 cc. of alcohol were added solutions of 10 gm. of potassium acetate in 55 cc. of alcohol and 10 gm. of semicarbazide hydrochloride in 130 cc. of water. The mixture was allowed to stand 48 hours at 38–40°. It was then concentrated under reduced pressure until practically dry. The residue was warmed with a little water for removal of salts, and then collected with water. After recrystallization from dilute alcohol, it melts at 305° after preliminary darkening. The yield was 9 gm.

3.662 mg. substance: 2.480 mg. H_2O, 8.105 mg. CO_2.
3.805 " " : 0.297 cc. N_2 (24.5°, 760.2 mm.).
$C_{24}H_{36}O_7N_3$. Calculated. C 60.34, H 7.40, N 8.80
  Found. (a) " 60.36, " 7.58
    (b) N 8.97

Desoxo-α-Isostrophanthidic Acid (*Isoperiplogenic Acid*)—2 gm. of α-isostrophanthidic acid semicarbazone were placed in a dry bomb tube with 50 cc. of absolute alcohol. To this were added 1.5 gm. of sodium. After solution of the sodium the tube was sealed and heated at 180° for 16 hours. The contents of the tube were washed out with water, and the solution was just acidified to Congo red and then allowed to stand in order to permit relactonization. At this stage a portion of the separating solid formed a resinous mass and was removed on a stirring rod and then dissolved in slightly diluted acetone. The solution was just acidified to Congo red and after standing an hour to insure complete lactonization was carefully diluted and the partly crystalline deposit was collected and joined with the main portion. It was then recrystallized by careful dilution of a methyl alcoholic solution. After repeated recrystallizations it melted at 215–217° with decomposition. The substance in ammoniacal solution did not decolorize permanganate. In sulfuric acid it formed a deep amber-colored solution, changing to red with an olive reflex on standing. The substance crystallized with approximately 1 mol of water of crystallization which was removed completely only on drying at 110° and 15 mm.

$[\alpha]_D^{24} = -23.3°$ (c = 1.056 in alcohol).
4.803 mg. substance: 3.490 mg. H_2O, 11.905 mg. CO_2.
$C_{23}H_{34}O_6$.   Calculated.   C 67.94, H 8.45
                     Found.   " 67.60, " 8.13

14.760 mg. of anhydrous substance were treated with 1 cc. of alcohol and titrated directly against phenolphthalein with 0.1 N NaOH.   Calculated for 1 equivalent for $C_{23}H_{34}O_6$, 0.363 cc. Found, 0.352 cc.

3 cc. of 0.1 N NaOH were then added and the solution was refluxed for 4 hours and then titrated back.   Calculated for 1 equivalent, 0.363 cc.   Found, 0.375 cc.

α-Isostrophanthidolic Methyl Ester—A solution of 1 gm. of α-isostrophanthidic acid in 65 cc. of methyl alcohol together with 20 cc. of HCl (1.19) was turbined with 25 gm. of amalgamated granulated zinc (30 mesh), during which operation the temperature was kept below 30°.   After 1.5 hours the cooled solution was decanted and then most of the free acid was neutralized with alkali. The concentrated solution was then extracted with chloroform. The latter was washed with sodium carbonate solution.   The residue obtained on evaporation of the solvent crystallized under methyl alcohol.   On recrystallization from methyl alcohol the substance separated as leaflets which melted at 223°.

$[\alpha]_D^{24} = -18.5°$ (c = 1.24 in pyridine).
4.992 mg. substance: 3.693 mg. H_2O, 12.126 mg. CO_2.
4.653 "   "   : 3.410 "   "   11.260 "   "
       $C_{24}H_{36}O_7$.   Calculated.   C 66.01, H 8.32
                     Found.   (a) " 66.24, " 8.28
                              (b) " 65.99, " 8.20

For comparison the rotations of α-isostrophanthidic acid and its methyl ester were also taken in pyridine and gave respectively $[\alpha]_D^{20} = -14°$ (c = 1.03 in pyridine) and $[\alpha]_D^{20} = -15°$ (c = 1.01 in pyridine).

γ-Isostrophanthidolic Methyl Ester—1 gm. of α-isostrophanthidic acid dissolved in a mixture of 100 cc. of methyl alcohol and 15 cc. of HCl (1.19) was refluxed with 25 gm. of amalgamated zinc. After 40 minutes a further 15 cc. of acid were added and the mixture was heated for 35 minutes more.   The reaction product was isolated as in the previous instance, but the yield of crystalline product was considerably less.   The chloroform residue only

partly crystallized under methyl alcohol. After recrystallization from this solvent, 0.17 gm. of sparingly soluble needles was obtained, which melted at 229–231°.

$[\alpha]_D^n = +98°$ (c = 1.000 in pyridine).
4.315 mg. substance: 3.225 mg. H_2O, 10.480 mg. CO_2.
4.105 "   "   : 2.260 " AgI.
$C_{24}H_{36}O_7$. Calculated. C 66.01, H 8.32, OCH_3 7.14
        Found.   " 66.24, " 8.35
                    OCH_3 7.27

Acetyl Isostrophanthidic Methyl Ester.—2.5 gm. of isostrophanthidic methyl ester were covered in a distilling flask with 50 cc. of acetyl chloride. After a few minutes a rather vigorous reaction occurred with complete solution. The excess of reagent was removed under diminished pressure, and the residue was dissolved in methyl alcohol. The acetate deposited as prisms on careful dilution. It was further recrystallized from ethyl alcohol, and melted with decomposition at 156–157°. The substance possesses a variable melting point. Different preparations melted between 127° and 157°.

$[\alpha]_D^{22} = -16°$ (c = 0.907 in pyridine).
4.040 mg. substance: 2.760 mg. H_2O, 9.680 mg. CO_2.
        $C_{26}H_{36}O_8$. Calculated. C 65.51, H 7.62
                Found.   " 65.35, " 7.64

12.510 mg. of substance were covered with 1 cc. of alcohol and 3 cc. of 0.1 N NaOH. The mixture was refluxed for 4½ hours and was then titrated back against phenolphthalein. Calculated for 3 equivalents, 0.788 cc. Found, 0.810 cc.

Acetyl-α-Isostrophanthidolic Methyl Ester—1.0 gm. of the acetate of isostrophanthidic methyl ester was hydrogenated in ethyl alcohol with 0.2 gm. of platinum oxide catalyst. A slow, steady absorption of 1 mol of hydrogen occurred, which required about 4 days for completion. The filtered solution was concentrated to dryness. The methyl alcoholic solution on careful dilution deposited cubes which sintered at 125–126° and melted with decomposition at 145°.

$[\alpha]_D^{22} = -25°$ (c = 1.22 in pyridine).
3.740 mg. substance: 2.645 mg. H_2O, 8.720 mg. CO_2.
        $C_{26}H_{38}O_8$. Calculated. C 65.23, H 8.00
                Found.   " 64.97, " 7.98

Sulfite of Isostrophanthidolic Methyl Ester—When the attempt was made to substitute the hydroxyl group of the above acetate with chlorine by means of thionyl chloride, a halogen-free substance was obtained, which proved to be the neutral sulfite. 0.1 gm. of the acetate was treated with 2 cc. of $SOCl_2$ at 0°. Immediate solution with effervescence occurred. After 45 minutes the excess reagent was removed under diminished pressure. The sulfite formed sparingly soluble crystals from methyl alcohol and melted at 220°.

4.557 mg. substance: 2.860 mg. H_2O, 9.968 mg. CO_2.
$C_{24}H_{34}O_8S$. Calculated. C 59.71, H 7.11
Found.   " 59.66, " 7.02

Sulfite of α-Isostrophanthidic Methyl Ester—0.2 gm. of α-isostrophanthidic methyl ester was dissolved in 4 cc. of $SOCl_2$ at 0°. After removal of the excess reagent the ester became crystalline under acetone. When recrystallized from methyl alcohol it formed needles which melted at 228°.

$[\alpha]_D^{24} = -40°$ (c = 0.56 in pyridine).
3.829 mg. substance: 2.302 mg. H_2O, 8.374 mg. CO_2.
$C_{24}H_{32}O_8S$. Calculated. C 59.96, H 6.71
Found.   " 59.64, " 6.72

STROPHANTHIN

XXI. THE CORRELATION OF STROPHANTHIDIN AND PERIPLOGENIN

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In a former communication,[1] the results were presented of an investigation on the cardiac glucosides which occur in *Periploca græca*. It was shown that by enzymatic cleavage of the glucosides present in a purified extract of this plant it is possible to obtain a chloroform-soluble substance, periplocymarin, which is a glucoside of the aglucone, periplogenin, with a methyl ether desoxy sugar, $C_7H_{14}O_4$. Periplogenin was found to possess the formula $C_{23}H_{34}O_5$ and to be a trihydroxytetracyclic-$\Delta^{\beta,\gamma}$-lactone. These facts at once suggested the probability of a close structural relationship with strophanthidin. This has now been substantiated by our more recent investigations.

In the preceding paper[2] the conversion of α-isostrophanthidic acid into its desoxo derivative has been described. This acid, $C_{23}H_{34}O_6$, has now proved on direct comparison to be identical with our previously described isoperiplogenic acid. This conclusion is supported by the results of a further comparison of the *methyl esters* of the acids obtained from both sources and of their oxidation products, α-*isoperiplogonic methyl ester* (*desoxo-α-isostrophanthido-nic methyl ester*).

Isoperiplogenic acid is, therefore, desoxoisostrophanthidic acid and the same relationship can be directly assumed to hold for the parent aglucones. Periplogenin is, therefore, desoxostrophanthidin in which the aldehyde group of strophanthidin is replaced by methyl. Their partial formulæ may be given as follows:

[1] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 519 (1928).
[2] Jacobs, W. A., Elderfield, R. C., Grave, T. B., and Wignall, E. W., *J. Biol. Chem.*, **91**, 617 (1931).

```
        CHO                                          CH₂
         |      OH^III                                |      OH
         CH     C                                     CH     C
        /  \   /H\                                   /  \   /H\
CH₂—C——CH   CH   CH₂              CH₂—C——CH   CH   CH₂
 |   ‖    |    |   |                      |   ‖    |    |   |
 CO  CH  COH^I COH^II CH           CO  CH  COH  COH  CH
  \ /      \  /                          \ /     \  /
   O        CH                            O       CH
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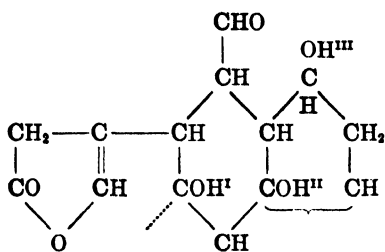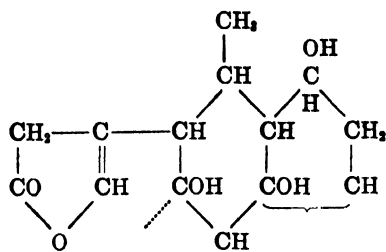
Strophanthidin   Periplogenin

We have in the meantime been able to procure more periplocymarin for an investigation of its sugar. This has been made possible through the kind cooperation of Doctor E. S. London of Leningrad, as well as Professor S. T. Petcheff of Suchum, Caucasus, and Doctor W. W. Stein of Sotshi, Caucasus, who arranged for the collection of a generous quantity of the plant *Periploca græca*. The sugar was found to be identical with cymarose, the methyl ether desoxy sugar which occurs in cymarin. Periplocymarin is therefore desoxocymarin.

<div align="center">EXPERIMENTAL</div>

α-Isoperiplogenic Acid (Desoxo-α-Isostrophanthidic Acid)—In the previous report of our analytical experience with this acid prepared from periplogenin the results given were obtained after drying at 100° and 20 mm. and indicated the retention of 0.5 mol of water in the dried samples. Following the results obtained with desoxoisostrophanthidic acid as given in the preceding paper, isoperiplogenic acid was similarly dried at 110° and 20 mm. and now gave satisfactory results.

4.437 mg. substance: 3.270 mg. H_2O, 11.005 mg. CO_2.
$C_{23}H_{34}O_6$.   Calculated.   C 67.94, H 8.44
                Found.   " 67.64, " 8.25

The acids from both sources melted at 215–217° with decomposition and a mixture of the two showed no depression. The crystalline form, six-sided or diamond-shaped leaflets, was identical in both cases, as were other physical properties.

α-Isoperiplogenic Methyl Ester (Desoxo-α-Isostrophanthidic

Methyl Ester)—The methyl esters prepared from the acids by means of diazomethane in acetone solution were found to be identical. In each case recrystallization from acetone gave long prisms which melted at 242° (not 252° as previously reported for the periplogenin derivative). The mixture showed no depression. For the strophanthidin derivative $[\alpha]_D^{23} = -30.2°$ (c = 1.110 in pyridine). For the periplogenin derivative $[\alpha]_D = -33.2°$ (c = 0.965 in pyridine).

The analysis of the ester from periplogenin has already been recorded. That obtained with the strophanthidin derivative is as follows:

4.200 mg. substance: 3.255 mg. H_2O, 10.580 mg. CO_2.
5.783 "   "   : 3.338 " AgI.
$C_{24}H_{36}O_6$. Calculated. C 68.51, H 8.64, OCH_3 7.38
        Found.   " 68.70, " 8.67
                        OCH_3 7.62

16.593 mg. of the ester of strophanthidin origin were refluxed for 4.5 hours in a mixture of 1 cc. of alcohol and 3 cc. of 0.1 N NaOH and then titrated back against phenolphthalein. Calculated for 2 equivalents, 0.788 cc. Found, 0.776 cc.

α-Isoperiplogonic Methyl Ester (Desoxo-α-Isostrophanthidonic Methyl Ester)—A solution of 0.5 gm. of desoxoisostrophanthidic methyl ester in 15 cc. of acetic acid was treated with 2.5 cc. of Kiliani's CrO_3 solution. After 10 minutes, dilution with water gave a crystalline precipitate. Recrystallized from methyl alcohol, the keto ester separated as sparingly soluble needles which melted at 228°.

$[\alpha]_D^{24} = -23°$ (c = 0.825 in pyridine).
3.905 mg. substance: 2.778 mg. H_2O, 9.872 mg. CO_2.
        $C_{24}H_{34}O_6$. Calculated. C 68.85, H 8.20
              Found.   " 68.95, " 7.96

The identical keto ester was similarly prepared from isoperiplogenic methyl ester. It melted at 230° and showed no depression when mixed with the above oxidation product.

$[\alpha]_D = -23.2°$ (c = 1.145 in pyridine).
4.245 mg. substance: 3.020 mg. H_2O, 10.742 mg. CO_2.
        Found. C 69.01, H 7.96

Cymarose from Periplocymarin—3 gm. of periplocymarin were shaken with a mixture of 21 cc. of alcohol, 13.5 cc. of water, and 6.7 cc. of HCl (1.19) until dissolved. The solution was left then at 20° for 3 hours. On dilution periplogenin separated. This was completed by allowing the mixture to stand at 0°. The filtrate was neutralized with an excess of Ag_2CO_3 and the Cl-free solution was treated with H_2S. The sugar solution after concentration to about 50 cc. was extracted with chloroform to remove impurities. The concentration to dryness was then completed. The residue was extracted with anhydrous ether and the ether solution of the sugar after partial concentration was diluted with petrolic ether. On seeding with cymarose the sugar separated slowly as beautiful long needles. After recrystallization from ether-petrolic ether the sugar melted at 100–102°, after considerable preliminary softening. This melting point was obtained, however, only after drying for several days over $CaCl_2$. Short exposure to moist air at once depressed the melting point. This explains the lower melting point (93°) previously recorded.[3] No depression in melting point was found on mixing the cymaroses from both sources.

$[\alpha]_D^{20} = +52°$ (c = 1.000 in water).
3.696 mg. substance: 2.860 mg. H_2O, 7.042 mg. CO_2.
3.862 "　　　" : 5.565 " AgI.
　　　　$C_7H_{14}O_4$.　Calculated.　C 51.81, H 8.70, OCH_3 19.13
　　　　　　　　　　Found.　　" 51.96, " 8.66
　　　　　　　　　　　　　　　　　　　　OCH_3 19.04

Addendum—In more recent work digitoxigenin and therefore gitoxigenin have been correlated with strophanthidin and periplogenin. Isoperiplogonic methyl ester which has just been described in these pages on dehydration loses OH^{II} and the resulting *anhydroisoperiplogonic methyl ester* on hydrogenation yields a mixture of isomeric desoxy derivatives. One of these has been found to be identical with isodigitoxigonic methyl ester in melting point, rotation, and crystalline form. This was confirmed by a further comparison of the desoxyisoperiplogonic acid obtained on saponification of the ester with isodigitoxigonic acid. Digitoxigenin is therefore desoxyperiplogenin and desoxodesoxystrophanthidin. The details of this work will soon follow.

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[3] Jacobs, W. A., *J. Biol. Chem.*, **88**, 527 (1930).

A CORRECTION CONCERNING THE HYPOGLYCEMIC ACTION OF p-AMINOPHENYLGUANIDINE HYDROIODIDE

BY THOMAS B. PARKS AND CHARLES E. BRAUN

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It was reported (1) as a result of a preliminary investigation, that p-aminophenylguanidine hydroiodide would produce a hypoglycemia in normal rabbits when administered by subcutaneous injection. Further investigation, not only of the hydroiodide salt, but also of the sulfate (2) and hydrochloride (2) salts of p-aminophenylguanidine, has produced results which lead to the conclusion that this guanidine derivative is not physiologically active. These observations are in agreement with the hypothesis that the aromatic nucleus is not productive of hypoglycemia (3).

Sixteen series of experiments were carried out with 63 normal rabbits as the experimental animals. The technique employed was essentially the same as that used in the preliminary investigation (1). Four different preparations of the hydroiodide, three of the sulfate, and one of the hydrochloride salt of p-aminophenylguanidine were tested for hypoglycemic action. Of the samples investigated the sulfates, the hydrochloride, and two of the hydroiodide salts produced neither hyperglycemia nor hypoglycemia, but two of the hydroiodide salts showed decided hypoglycemic properties, as has been reported (1), with maximum lowering of the blood sugar occurring in from 4 to 6 hours following injection. Representative data are recorded in Table I.

It was observed that the crystals of the hydroiodide salts which appeared to have hypoglycemic properties were not pure white but had a slight brownish tint. (These had been recrystallized from 95 per cent ethyl alcohol.) On the other hand, the inactive hydroiodide salts and the three sulfate salts were white, while the

TABLE I

Effect of Hydroiodide, Sulfate, and Chloride of p-Aminophenylguanidine upon Blood Sugar

Compound	Preparation No.	Dose	Concentration of solution injected	Blood sugar readings, mg. per 100 cc.								
				Initial	1½ hrs.	2 hrs.	3 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	24 hrs.
		mg. per kg.	*mg. per cc.*									
$H_2NC_6H_4N_3CH_4\cdot HI$	1	10.0	10.0	116	99		86		62		80	96
		30.0	10.0	103	99		97		71		85	87
		50.0	10.0	107		104	98		69		80	95
	2	10.0	10.0	78		79		76	71			82
		20.0	10.0	96		96			102	101		100
		75.0	10.0	99		99			104	103		96
	3	5.0	2.5	100		85		76	74	77		89
		5.0	2.5	108		86		85	76	77		94
		5.6	2.5	91		82		74	80	87		
		5.6	2.5	83		74		68	72	80		
		10.0	5.0	112		94		70	94	99		100
		10.0	5.0	116		99		70	98	100		109
	4	5.6	2.5	97		100		99	98	100		
		5.6	2.5	93		90		91	92	90		
		5.6	2.5	94		92		93	95	94		
		10.0	2.5	104		99		101	102	100		
		10.0	5.0	96		92		90	95	94		
$H_2NC_6H_4N_3CH_4\cdot\tfrac{1}{2}H_2SO_4$	1	5.0	2.5	88		82		85	84	86		
		5.0	2.5	84		86		87	85	86		
		10.0	2.5	92		90		91	89	90		
		10.0	2.5	89		87		88	91	89		
	2	10.0	2.5	87		84		85	87	88		
		50.0	10.0	111		108		109	111	110		
		100.0	30.0	102		104		106	108	107		
	3	50.0	10.0	100		98		97	99	100		
		100.0	30.0	107		103		102	104	108		
$H_2NC_6H_4N_3CH_4\cdot HCl$	1	3:7	2.5	95		89		91	90	92		
		3.7	2.5	96		88		90	87	89		
		10.0	5.0	94		89		90	91	96		
		10.0	5.0	89		85		84	91	90		
		20.0	5.0	94		92		90	88	89		

hydrochloride salt had a very slight trace of yellow in it. (The latter salts had been recrystallized from water.) In an attempt to explain the inconsistent physiological results, it occurred to us that perhaps the slight discoloration was related to the apparent hypoglycemic activity of the two hydroiodide salts as compared with the physiological inactivity of the white compounds, and that this discoloration might have been caused by a slight oxidation. Therefore, some of the inactive preparations were oxidized by heating them in aqueous solutions exposed to the air for varying periods of time. The solutions thus treated discolored, but when tested physiologically produced no hypoglycemia.

Another attempt was made to explain the perplexing physiological results. It was thought that perhaps the hypoglycemic action of the two hydroiodide salts referred to above was due to an impurity present in these compounds in amounts too small to be detected in standard analytical procedure.

Analytical data on these compounds were as follows:

		Found.	Calculated.
Sample 1.	Iodine (as AgI)	45.44	45.65
" 3.	" " "	45.47	45.65

Sample 3, a hydroiodide salt which had given some indications of hypoglycemic properties, was dissolved in distilled water and fractionally recrystallized under a vacuum over concentrated sulfuric acid at room temperature, in an attempt to isolate or to concentrate in one fraction the minute trace of any impurity which might be causing the physiological results referred to above.

During the recrystallization no mother liquor nor solid was ever discarded, so that the only loss of material which possibly could have occurred was that in the solutions absorbed by the filter papers. Four fractions were obtained; their melting points, colors, and physiological activities were as shown on following page.

While the Folin (4) micro method for blood sugar appears to give low results, the erratic physiological observations shown in Table I for the hydroiodide salt are probably not due to an error in method. This is indicated by the fact that the physiological inactivity of Sample 4 was confirmed by a series of experiments in which the blood sugar was determined by the method of Shaffer and Hartmann (5).

Substance	M.p.	Color	Physiological action
	°*C*.		
Hydroiodide salt,Sample 3	209 –211	White-brown tint	See Table I
Fraction 1	210 –211	White	Inactive
" 2	209.5–211 (Not as sharp as Fraction 1)	Yellowish brown	"
" 3	209 –211	Light brown	"
" 4 (Obtained by evaporating mother liquor from Fraction 3 to dryness under a vacuum)	Began to melt at 180°, complete at 201°	Orange-brown	"

Although no explanation is offered for the inconsistent physiological results of the four hydroiodide salts, nevertheless, in view of the consistent inactivity shown by the three sulfate salts and the hydrochloride salt, together with the fact that the hydroiodide failed to produce a consistent hypoglycemia, it is concluded that *p*-aminophenylguanidine in the form of its inorganic salts does not possess hypoglycemic properties. It is significant in this connection to note that Bischoff, Sahyun, and Long (3) encountered a similar experience when investigating the hypoglycemic action of hydroxymethylcyanoguanidine.

The authors wish to acknowledge with thanks the cooperation of Professor Ernest L. Scott of the Department of Physiology, College of Physicians and Surgeons, Columbia University, who very kindly checked the results on the hydroiodide salt, Sample 4. Thanks are due to Hazel Tedford Parks for her assistance in the animal experimentation.

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A STUDY OF CERTAIN PROPERTIES OF THE PROVITAMIN A*

By E. J. QUINN and J. G. HARTLEY

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A continuation of the studies conducted in this laboratory to determine the properties of the vitamin A factor from plant tissue has given information regarding the behavior of this factor toward certain adsorbents and organic solvents, which is believed to be of considerable interest in view of the recent findings regarding the relationship of carotene to vitamin A.

In the present investigation petroleum ether extracts of the dry carrot root have served as the source material of the plant factor to be studied. These clear, highly pigmented solutions have been found to contain an abundance of vitamin A or the provitamin, for, when the extracts dried on corn-starch were tested according to the method of Sherman and Munsell (1) and Sherman and Burtis (2), 0.25 gm. of the extract-starch residue (50 cc. of the extract dried on 20 gm. of corn-starch) fed daily produced good growth in the experimental animals during an 8 weeks experimental period.

Upon treating the carrot extracts with norit or Lloyd's reagent the color was found to be readily removed from solution, 2.5 gm. of the former and 8.5 gm. of the latter adsorbent completely decolorizing 50 cc. of the yellow extract, the color of which corresponded to that of a $K_2Cr_2O_7$ solution containing 2.0 to 4.0 mg. of this salt per cc.

When the colorless filtrates obtained on filtration of the adsorbents were tested for vitamin A with the Carr and Price (3) reagent or when they were fed to animals on a vitamin A-free basal diet, negative results for this vitamin were obtained in each case. Since

there is considerable evidence that the vitamin A of liver oils is not adsorbed by norit, the negative results in the above tests indicated the absence in the filtrates of a second growth-promoting factor analogous to the vitamin A commonly found in cod liver oil.

With the plant pigment from the petroleum ether extract adsorbed on the norit or Lloyd's reagent, a study was next made to determine whether the active factor so adsorbed could be removed by various organic solvents. Among the reagents tried were chloroform and ethyl ether and it was discovered that while the chloroform very readily removed the pigment adsorbed on the norit it dissolved very little of the color from the Lloyd's reagent residue. Just the reverse of this followed the use of ethyl ether, this solvent removing very little color from the norit residue but a considerable quantity from the Lloyd's reagent residue.

A rather unexpected result was obtained on testing the highly pigmented chloroform extract of the norit residue and the equally colored ethyl ether extract of the Lloyd's reagent residue when these materials were fed to animals prepared for vitamin A tests. Examined with the Carr and Price antimony trichloride reagent, each of the extracts gave a blue color which, if we assume this blue color change to be due to the presence of vitamin A or its precursor, indicated that the extracts would be potent in the feeding tests. It was found, however, on feeding the solutions dried upon corn-starch that the materials contained little if any vitamin.

The reason for the lack of potency was suspected when it was noticed that the colored residues obtained by evaporating the chloroform or ethyl ether extracts on corn-starch lost their yellow color within 2 or 3 days after the samples were prepared, the change occurring in spite of the precautions of using covered, amber glass containers and storing the materials at a low temperature. This experience now appears to be similar to that reported by Drummond, Ahmad, and Morton (4) in their attempts to use ethyl oleate as a solvent for carotene. While it is probable that oxidation was largely responsible for the loss of pigment experienced, it is significant that petroleum ether extracts of carrots dried on corn-starch and stored under similar conditions retained for several weeks both their color and vitamin A activity.

Holm (5) has advised against the use of ethyl ether as a solvent in vitamin work. According to the present study chloroform

should certainly be avoided as a solvent for the provitamin A. To determine the relative rapidity with which some of the solvents used here affected the yellow pigment dissolved therein, extracts of carrots made with (a) chloroform, (b) ethyl ether, and (c) petroleum ether were stored in stoppered test-tubes, one set exposed to the light from a north window, the other stored in a dark room.

That light was a contributing factor in the destruction of the pigment of the solution was evident from the comparatively rapid deterioration or loss of color in the chloroform extract kept in the light compared with the small change occurring in a portion of the same sample stored in the dark. Of the three samples exposed to the light the color of the chloroform extract was affected most seriously, that of the ethyl ether very slowly (no change in the dark), while the petroleum ether extracts remained practically unchanged during the several weeks of observation.

Since it has been found that peanut oil readily dissolves and extracts the vitamin A factor from dried plant tissue, it was desired to determine whether this oil would also extract the active factor adsorbed on norit and Lloyd's reagent. To determine this, 50 cc. portions of petroleum ether extracts of carrots (color equivalent to 2 to 4 mg. per cc. of $K_2Cr_2O_7$ standard) were decolorized with 2.5 gm. of norit or 8.5 gm. of Lloyd's reagent. The norit and Lloyd's reagent residues thus obtained were filtered, washed three times with 5.0 cc. of fresh petroleum ether, then dried, and each extracted with 30 cc. of peanut oil. The oil extracts were filtered through quantitative paper and tested for their vitamin A potency.

As will be seen from Fig. 1, rats fed 1.5 gm. of the oil extract of norit residue weekly (0.5 gm. three times per week) made excellent growth, but the animals given the same amount of the oil extract of Lloyd's reagent residue lost weight and their survival was approximately the same as the negative controls and those receiving peanut oil only as supplements to the basal diet.

The question arose during the progress of these experiments whether the vitamin, which appeared so securely held by the adsorbents as not to permit of its removal by certain solvents, would be of any value to the animal body if fed in this adsorbed condition. Frequently in vitamin work, especially in attempts at concentration of the vitamin, the active factor is removed from solution by an adsorbent and the adsorbed residues thus obtained fed to ex-

perimental animals for the purpose of testing the potency of the materials under investigation. In all probability the water-soluble vitamins would be removed from such adsorbents during digestion but it was thought that the fat-soluble vitamin with which we were concerned might act differently. To determine whether this were the case the norit and Lloyd's reagent residues prepared as has been described were mixed with the vitamin
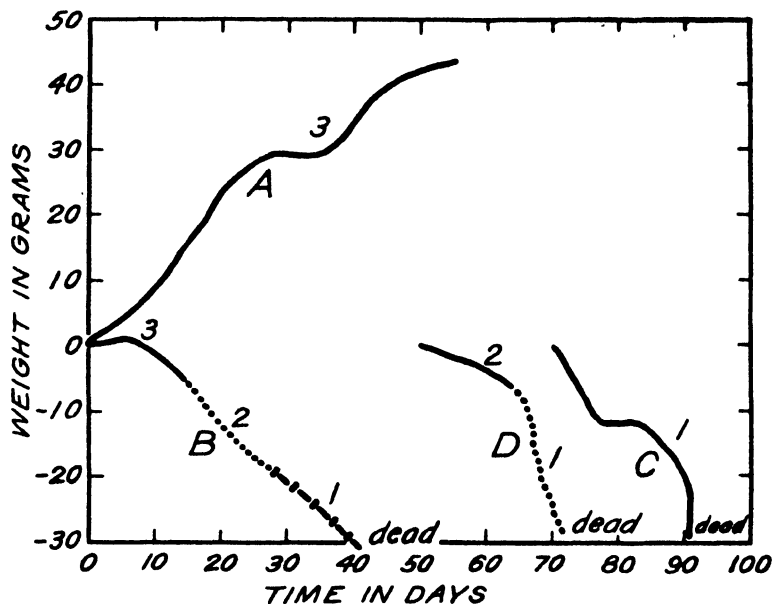
FIG. 1. Growth curves of animals fed, in addition to the vitamin A-free basal diet, one of the following supplements: (A) Peanut oil extract of vitamin A factor adsorbed on norit. 0.5 gm. fed three times per week. (B) Peanut oil extract of vitamin A factor adsorbed on Lloyd's reagent. 0.5 gm. fed three times per week. (C) Peanut oil. 0.5 gm. fed three times per week. (D) Basal diet only. The numbers on the curves are the number of animals represented.

A-free (and fat-free) basal diet and fed to rats at a level which, providing the active factor were removed by the animal, would furnish sufficient amounts of the vitamin to produce good growth.

As shown in Fig. 2, the animals receiving norit residue (1.5 gm. weekly) were able to remove and utilize at least part of the vitamin that was adsorbed, for there was a definite growth response follow-

ing the feeding of this material.   The growth was not at all what was expected, however, in view of the better results obtained when equivalent amounts of the vitamin factor were fed in the form of the original petroleum ether extract of carrots or the peanut oil extract of the norit residue.

Animals furnished the Lloyd's reagent residue (4.8 gm. weekly) were evidently unable to obtain much of the vitamin from this ad-
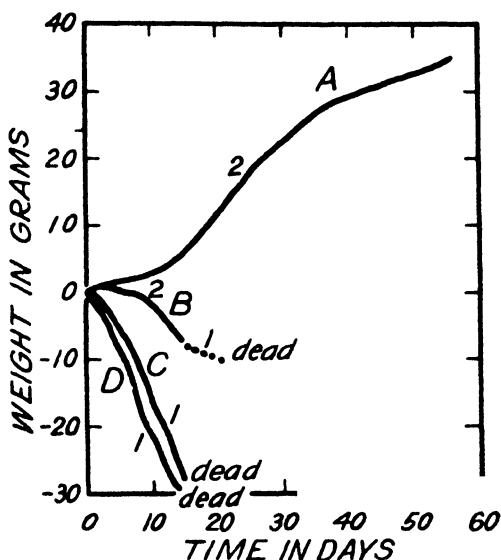
FIG. 2. Growth curves of animals fed, in addition to the vitamin A-free basal diet, one of the following supplements: (A) Norit containing the adsorbed vitamin A factor from carrots. 0.5 gm. fed three times per week. (B) Lloyd's reagent containing the adsorbed vitamin A factor from carrots. 1.6 gm. fed three times per week. (C) Norit. 0.5 gm. fed three times per week. (D) Lloyd's reagent. 1.6 gm. fed three times per week. The numbers on the curves are the number of animals represented.

sorbent, for their behavior during the test period was quite like that of negative controls.

Supplementary to the above experiment, to determine how effectively the animals used the vitamin adsorbed on norit or Lloyd's reagent, the feces of the animals fed these adsorbed residues were extracted with a suitable solvent and the color of the

filtered solutions compared with extracts of the feces of control animals receiving untreated norit or Lloyd's reagent.

Chloroform extracts of the feces from the animals fed norit residue were highly pigmented and gave a light blue color upon the addition of antimony trichloride, while the feces of animals receiving norit which carried no adsorbed pigment were practically colorless and gave a faint red color with the Carr and Price reagent. It is concluded that, although the animal was able to remove some of the adsorbed vitamin, part of that which was fed passed through the alimentary tract without being absorbed.

Ethyl ether extracts of feces from the animals receiving the Lloyd's reagent residue were but slightly colored and gave no blue color with the antimony trichloride. Since the pigment carried on the Lloyd's reagent was apparently not utilized by the animal and did not appear to any measurable extent in the feces, it would seem that the factor must have been destroyed or changed to an insoluble form at some stage of the test period.

SUMMARY

The provitamin A may be readily adsorbed from petroleum ether extracts of dried carrots by both norit and Lloyd's reagent.

The adsorbed pigment can be removed very readily from the norit by extraction with chloroform but very little appears to be dissolved by ethyl ether. Ethyl ether removes more of the adsorbed color from Lloyd's reagent residue than does chloroform.

Due to the unstable nature of the pigment in chloroform solution, the desirability of using this solvent in work of this kind is questioned.

The vitamin factor adsorbed on norit is readily dissolved and extracted by peanut oil. This oil was found, however, to extract little or none of the potent factor adsorbed on Lloyd's reagent.

It was found that rats fed norit containing the adsorbed vitamin were able to remove and utilize some of this vitamin. Rats were apparently unable to extract any of the factor which had been adsorbed on Lloyd's reagent. There is a possibility, however, that the vitamin that has been adsorbed on this last named adsorbent was destroyed, perhaps by oxidation, at some stage in the test period.

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THE PREPARATION OF THEELOL*

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In an earlier paper (Veler, Thayer, and Doisy, 1930) we have described a process for the extraction of theelin which in our hands has never failed to yield the crystalline product in perhaps forty or fifty consecutive preparations. However, one annoying circumstance has been encountered; namely, the large and variable proportion of rat units that is not extracted from alkaline solutions by ethyl ether. To secure greater clarity a sketch of the procedure given in that previous publication (p. 370) is included here.

Though a loss occurs in Step 4, we have focussed our attention thus far on Step 7. It was found that after extraction with ether the alkaline solution of Step 7 contained a large proportion of the estrogenic substance. Upon acidification a precipitate which was partially soluble in ether usually formed. A test of this solution with spayed rats showed it to be active. Various procedures were instituted to obtain pure crystalline theelin but without success. The estrogenic substance of this fraction seemed to differ from theelin.

Since we (Doisy, Thayer, Levin, and Curtis, 1930) have isolated two estrogenic substances it would not seem desirable to retain any of the older names which were in use before the existence of two distinct compounds was known. Thus, folliculin more or less commits us to the idea that the estrogenic substances of pregnancy urine are identical with that of the liquor folliculi. Oestrin or estrin was the name given to the substance that provoked estrus. As we know now there are two such substances, to each of which identifying names should be given.

1. Urine; butyl alcohol extraction in continuous liquid extractor

2. Butyl alcohol distilled; residue leached with benzene

2 a. Solution   2 b. Insoluble residue discarded;
                                       3.2 per cent loss

3. Distilled and residue transferred to solution in dilute NaOH

3 a. Alkaline solution   3 b. Insoluble tar discarded; 4.2
                                       per cent loss

4. Extracted with ethyl ether

5. Ether distilled; residue steam-distilled

6. Residue leached with hot 0.25 N NaOH

6 a. Solution   6 b. Residue discarded; 1 per cent
                                       loss

7. Extracted with ethyl ether; ether distilled

8. Residue leached with cold 0.25 N NaOH

8 a. Solution extracted with ether;   8 b. Insoluble residue discarded;
     ether distilled   9 per cent loss

9. Residue crystallized from hot 25 per cent ethyl alcohol or butyl
   alcohol.

At the suggestions of Dr. Paul N. Leech and Dr. A. M. Schwitalla we called the compound first isolated theelin from the Greek, Theelus, meaning woman or female. We propose now, in view of the facts that the new substance is a trihydroxy compound and that it shows physiological and possibly chemical similarities to theelin, to name it theelol. The acknowledged necessity of names for new compounds and the appropriateness of the names suggested should warrant the general adoption of theelin and theelol.

Purification of Theelol

The alkaline solution of Step 7 is extracted with ethyl ether. Though the extraction of theelin by the ether is not complete subsequent steps remove it so that it does not contaminate the theelol. The alkaline solution is faintly acidified with HCl and gently heated to remove the ether and coagulate the precipitate. After cooling, the brown precipitate is filtered off with suction on a Buchner funnel, dissolved in 95 per cent ethyl alcohol, and transferred to a large beaker. The solution is slowly concentrated. If distilled, a tarry mass usually results and if a small beaker is used or too rapid evaporation occurs the solution creeps over the top of the beaker. After concentration as directed, a brown semicrystalline mass separates on cooling. This is filtered off with suction and recrystallized once or twice as described above. The crystals are then dissolved in 95 per cent ethyl alcohol; the solution is boiled with norit, cooled, filtered, and the filtrate concentrated. 3 volumes of distilled water are added with stirring. After standing for some time the nearly white precipitate is filtered off and recrystallized until colorless from redistilled 95 per cent ethyl alcohol. Two or three recrystallizations generally suffice. Due to the preliminary ether extraction and the resulting relative quantities of theelol and theelin, the recrystallizations from alcohol suffice to remove the latter satisfactorily.

Upon examination of this crystalline product it was found to differ from theelin in solubility, crystalline form, specific rotation, physiological activity, and chemical composition.

In only a few instances do we have even an approximate idea of the yield of theelol. From 110, 200, and 60 gallons of urine, we obtained 300, 810, and 328 mg. of pure crystals, thus giving from 3 to 5 mg. per gallon. In addition, a considerable quantity of the triol has been isolated from the combined alcoholic mother liquors. The figures given, then, are minimum values for a supply of mixed pregnancy urines.

The yield of theelin and theelol should be contrasted. As a rule we obtain between 0.5 and 1.0 mg. of the former per gallon of urine, which is from 0.1 to 0.2 of the yield of theelol. On the basis of 0.5 mg. of theelin and 5 mg. of theelol per gallon, we are obtaining 9000 (1500 + 7500) rat units in a crystalline form.

Evidence of Existence of Two Physiologically Active Compounds

One might argue as Marrian (1930) did, that the two crystalline products are themselves inactive but owe their activity to an adsorbed impurity. We believe that our evidence is difficult to interpret on this basis.

Briefly, the evidence may be summarized by stating that theelin is approximately twice as active as theelol in adult spayed rats, whereas theelol is six or seven times as active as theelin in immature female rats. Using spayed rats, assays of several preparations of theelin have given 3000 rat units per mg.; of theelol, 1500 rat units. With the opening of the vagina during a period of 10 days following the beginning of the injection of female rats 18 days of age as a criterion of activity, approximately 0.16γ of theelol suffices; of theelin, 1.08γ.

It would seem from the above paragraph that two different substances effective in producing changes in the genitalia of female rats must exist in the extracts of the urine of pregnant women. Such a conclusion seems to be a necessity. Whether we have isolated those substances is left for the reader of the accompanying papers to decide. We wish, however, to reiterate that in spite of our faith in our evidence, we realize that the absolute certainty of isolation does not exist until synthesis is effected.

DISCUSSION

By suitable additions to the procedure for the preparation of theelin, a new crystalline triol, theelol, has been isolated from the butyl alcohol extracts of pregnancy urine. It seems to possess greater dissociation as an acid than theelin yet in general its properties are very much the same. Judging from the description of the procedure published by Marrian (1930), it would seem possible that his product is a mixture of theelin and theelol. In none of the steps given is an alkaline solution extracted· with an organic solvent, but in each case the alkali is neutralized with an acid (CO_2) before extraction with ether. This permits the extraction of both theelin and theelol. Possibly a fair proportion of theelin was eliminated by the precipitation of the alcoholic solution with ether and by the recrystallizations from organic solvents. However, the melting point, 264-266° (uncorrected) given is inter-

mediate between the melting points of theelin 249° (uncorrected) and theelol 274° (uncorrected) which might indicate that a mixture of the two was obtained.

The isolation of theelin by D'Amour and Gustavson (1930) seems to be satisfactory with respect to the elimination of theelol as alkaline solutions were repeatedly extracted with ethyl ether.

Neither Butenandt (1930) nor Laqueur (Dingemanse, deJongh, Kober, and Laqueur, 1930) has reported the occurrence of the triol, though both have prepared theelin. Since Butenandt's product agrees very closely with our theelin both in melting point and analysis, it is probably not contaminated with the triol. The low melting point of Laqueur's preparation (240°) indicates that it is not free from impurities.

SUMMARY

1. A process for the extraction of a new triol from the urine of pregnant women is described.

2. It is proposed that the name, theelol, be given to this triol.

3. Both theelin and theelol can be readily obtained in pure crystalline form by the process described.

We are indebted to Mr. Edward Smith for extensive assistance with the extractions and fractionations.

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THE BIOASSAY OF THEELOL*

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The isolation of a triol which seemed to show a chemical similarity to theelin led us to compare the physiological effects of the administration of the two compounds. Our experiments may be grouped together under two main headings: (1) the response of spayed adult females, and (2) the response of immature rats 18 to 21 days of age.

Assay with Adult Ovariectomized Rats

The assay of theelol was conducted by the procedure outlined by Kahnt and Doisy (1928). The total volume of solution was injected subcutaneously in three portions, at 9 a.m., 1 p.m., and 5 p.m. on 1 day. Smears were made 48, 52, 56, and 72 hours after the first injection. Twenty rats were injected with each dosage, the rat unit being the smallest quantity that produced a positive response in 75 per cent (15) of the rats.

With our routine procedure, some differences between theelin and theelol were discovered. The former seems to be approximately twice as active as the latter. Furthermore, the response to theelol seems to be more prolonged. In a large proportion of our rats, the smears did not return promptly to the negative condition.

With Preparation E 194, twenty rats gave a positive response with 0.65γ (1γ = 0.001 mg.), with 0.52γ eight out of twenty were positive. Preparation E 194 g which was the crystalline triol re-

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covered by the hydrolysis of the triacetyl derivative of Preparation
E 194 gave a positive smear in eighteen out of twenty rats injected
with 0.65γ; with 0.52γ fourteen out of the twenty were positive.
We conclude from these assays that these preparations assay
between 1500 and 2000 rat units per mg. Of many crystalline
preparations of theelin, two have assayed 4000, one 2500, and the
others 3000 rat units per mg. It is believed that these differences
in the assay of theelin are due to the limitations of this bio-
assay method and that for the purpose of comparison, it is safe
to say that theelol is one-half as active as theelin in the spayed
adult rat.

Assay with the Immature Female Rat

Since theelol seemed to differ from theelin, it seemed desirable to
ascertain whether the former influenced ovulation. In our earliest
work with the immature female it appeared that theelol caused
early ovulation, since several of our animals showed large follicles
or corpora lutea before they were 40 days of age. We now believe
this to have been fortuitous since in our later work with females
18 days old, the establishment of vaginal introitus has not been
connected with ovulation. It seems probable that the opening of
the vagina and the positive smears are due to the direct action of
theelol and are similar in nature to the response of the ovariecto-
mized female. The second positive smear is probably associated
with the first ovulation.

Normal Controls; Age of Opening of Vagina

Long and Evans (1922) state that the age of establishment of
vaginal introitus may vary a great deal. In our colony, 62 females
have been distributed as follows: eight from 35 to 39 days; twenty-
nine from 40 to 44 days; twelve from 45 to 49 days; and thirteen
from 50 to 55 days.

Since opening may occur at such an early age (35 days) we de-
cided to use animals 18 days old. Furthermore, it seemed to us that
age was an important factor in the quantity of theelol required to
produce opening. Our earlier data in which animals varying from
18 to 25 days old were used are not utilized in this paper because
of their irregularities. In a few instances less than 0.10γ caused
opening of the vagina in rats that were injected at an age of 23 or

24 days. We have, therefore, used animals that were 18 or 19 days of age in the hope of securing greater uniformity of results.

Administration in three portions on 1 day only seemed to produce irregular results. We have therefore used a subcutaneous injection in the morning and evening of 3 successive days beginning when the animal was 18 days old. In a few instances 19 day rats have been used. When sufficient rats were available five were injected with each volume. The unit tentatively adopted is defined as the minimum quantity that causes establishment of the vaginal opening within 10 days in three out of the five animals. The 10 day period makes the oldest age acceptable 1 week less than the

TABLE I

Comparison of Theelol and Theelin: Subcutaneous Administration

	Preparation E 194 (theelol)				Preparation E 199 (theelin)		
No. of rats used	Amount injected, γ	At start of injection	At opening of vagina	No. of rats used	Amount injected, γ	At start of injection	At opening of vagina
			Age of rats				Age of rats
		days	*days*			*days*	*days*
5	0.10	18	25, 29, 33, 26, 30	3	0.36	18	39, 42, 43
3	0.156	19	28, 28, 29	3	0.54	18	37, 39, 42
3	0.156	18	23, 24, 24	5	0.72	18	29, 33, 34, 34, 34
6	0.187	18	24, 24, 24	5	1.08	18	23, 23, 23, 23, 34
			25, 25, 27	3	1.62	18	23, 23, 23

age of the youngest control rat in which opening occurred. This unit may have no great significance but it seems from our observations that this method of assay assists in establishing the non-identity of theelin and theelol.

The data of Table I indicate that 0.16γ of theelol and 1.08γ of theelin are the immature rat units. These figures show that the former is 6 or 7 times as active as the latter when the establishment of vaginal introitus is taken as a criterion of activity. On the other hand the production of cornification in the spayed adult shows that theelin is twice as active as theelol. About ¼ of the spayed rat unit of theelol suffices to cause opening of the vagina of the immature rat; 3 spayed rat units of theelin are necessary.

Oral Administration

Though our data on oral administration are incomplete they are nevertheless of interest. Several preparations have been partially assayed; the data on Preparation E 194 being given in Table II. With the establishment of the vaginal orifice as a criterion of activity the rat unit of theelol seems to be 1.5 or 2.0 times the amount required subcutaneously; with theelin the oral and subcutaneous units are approximately equal.

The early work of Allen *et al.* (1924–25) led them to believe that the follicular hormone was not effective by enteral administration. Later work (Laqueur *et al.*, 1927) indicated that 100 times

TABLE II

Enteral Administration of Theelol (Preparation E 194)

| No. of rats used | Amount administered, γ | Age of rats | |
		At start of administration	At opening of vagina
		days	*days*
2	0.104	19	28, 35
2	0.208	19	26, 40
2	0.312	19	24, 26
3	0.312	18	23, 23, 25
2	0.416	18	23, 23

the subcutaneous unit given orally to the spayed rat was required to produce positive smears. We have administered theelol orally to spayed rats. Though the results are somewhat irregular, it seems that the unit enterally is from 2 to 3 times the subcutaneous unit.

With respect to the establishment of the vaginal orifice theelin is about as effective enterally as it is subcutaneously, thus indicating that it is absorbed in the young rat. The response of the spayed adult rat to the oral administration of theelin has not been studied.

SUMMARY

1. With subcutaneous injection of spayed rats, theelol assays at least 1500 rat units per mg.; orally it is from one-third to one-half as active.

2. With subcutaneous injection of 18 day female rats, theelol assays about 6000 rat units per mg.   With enteral administration the value falls to 3000 rat units per mg.

We are glad to record our indebtedness to Miss Corinne Dewes for her assistance with the assays.

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THE EFFECT OF THEELOL ON THE BLOOD PRESSURE, HEART RATE, AND RESPIRATORY RATE

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The isolation of the crystalline theelol has led to a study of the effect of this compound upon the blood pressure, heart rate, and respiratory rate of anesthetized dogs. In earlier work Laqueur (1927) found that menformone, a solution with estrogenic properties, did not influence the respiratory rate or blood pressure of dogs. However, the composition of menformone is uncertain. It would seem from Laqueur's later paper (Dingemanse, deJongh, Kober, and Laqueur, 1930) that the chief constituent is probably theelin. It is therefore desirable to record our observations on the pure theelol.

A solution of Preparation M 184 (melting point 273° uncorrected) was made by dissolving 0.491 mg. of this substance in 1 cc. of 0.25 N NaOH and diluting to 10 cc. with saline. The resulting solution was approximately isotonic, had an alkalinity of 0.025 N NaOH, and contained 0.0491 mg. of Preparation M 184 or approximately 100 rat units per cc. 2.5 cc. were injected intravenously. Blood pressure, heart rate, and respiratory rate were recorded before, during, and after injection. Nine experiments were performed on dogs ranging in weight from 8 to 16 kilos. Nembutal (No. 844, Abbott) was used as an anesthetic in six experiments, ether was used in three cases. No changes in the blood pressure, heart rate, or respiratory rate were observed other than those which occur spontaneously under anesthesia. Since no changes were observed it seems unnecessary to publish the records in detail.

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CHARACTERIZATION OF THEELOL*

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In a preliminary paper (Doisy, Thayer, Levin, and Curtis, 1930) we reported the isolation of a crystalline triatomic alcohol from the urine of pregnant women. The evidence available at that time led us to conclude that the new triol (subsequently named theelol) differed from the pregnandiol previously isolated by Marrian (1929) and Butenandt (1930, *a*) and from the crystalline estrogenic compound isolated by Doisy, Thayer, and Veler (1930), Butenandt (1930, *b*), and Laqueur (Dingemanse, deJongh, Kober, and Laqueur, 1930) and also from the new triol found by Marrian (1930).

Theelol can be differentiated from pregnandiol on the basis of physiological activity, since both Marrian and Butenandt report that the administration of large quantities of the diol does not cause estrus in spayed mice. Furthermore, the chemical composition is distinctly different (see Table I).

The differences between theelol and theelin (or Butenandt's progynon and Laqueur's menformone) are not so clear-cut. Both have the same number of carbon atoms per molecule but theelol is a triol whereas theelin is a monoatomic alcohol with its second oxygen atom in a keto linkage. In view of the chemical and physiological similarities it would seem probable that both are derivatives of the same mother substance.

With regard to the triol recently reported by Marrian, the comparison is a bit more difficult. The data given indicate that a

TABLE I

Comparison of Pregnandiol, Theelin, and Theelol

	Pregnandiol		Theelin		Marrian's triol	Theelol	Calculated $C_{18}H_{24}O_3$
	Marrian	Butenandt	Thayer and Doisy	Butenandt			
C, per cent..........	78.08	78.64	79.69	79.68	74.76	75.12	74.95
H, per cent..........	11.18	11.33	8.49	8.21	8.39	8.38	8.39
OH................	2	2	1	1	3	3	
C=O...............	0	0	1	1	0	0	
Molecular weight......	284	320*	270*	270*	277	295	288
Melting point........	233–234.5°	234–235°	248° (un-corrected)	250–251° (corrected)	264–266°	273.8°	
Iodine No...........	0	0	95.2			86.7	
Formula............	$C_{19}H_{32}O_2$ or $C_{20}H_{34}O_2$	$C_{21}H_{36}O_2$	$C_{18}H_{22}O_2$	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_3$	$C_{18}H_{24}O_3$	

* Calculated from the formula.

triol has been analyzed. However, the melting point is 8° lower than the melting point found for our compound. Since our data point rather conclusively to a triatomic alcohol it seems that two possibilities are open, *i.e.* either two isomeric triols have been isolated or Marrian's triol was not pure (also discussed in the paper by Doisy and Thayer, 1931) and therefore has a low melting point.

The data of Table I were compiled from the publications of the various investigators by the computation of average values where the results of more than one determination are given. It appears that three and possibly four different alcohols of high molecular weight have been isolated from pregnancy urine.

TABLE II

Elementary Composition of Theelol

Preparation No.	Weight of sample	CO₂	H₂O	C	H
	mg.	*mg.*	*mg.*	*per cent*	*per cent*
M 184*	5.450	15.020	3.995	75.16	8.20
E 194	4.208	11.590	3.180	75.11	8.45
M 200 c	3.809	10.489	2.885	75.10	8.47

* By Research Service Laboratories.

Theelol. Elementary Composition and Molecular Weight Determination by Rast's Camphor Method

Qualitative analysis (Table II) failed to detect sulfur, halogens, or nitrogen. Samples for carbon and hydrogen were dried in a desiccator over $CaCl_2 + P_2O_5$ and then heated in a vacuum oven (20 mm. Hg) for 3 hours at 105°.

Examination of the combustion data and molecular weight determinations (Table III) indicates a probable formula of $C_{18}H_{24}O_3$. Calculated, C 74.95; H 8.39; molecular weight 288.

Melting Point

The melting points of several samples (Table IV) were taken by the open beaker method with a 360° Bureau of Standards thermometer. A short stem calibrated thermometer was used to determine the melting point of two preparations; the corrected reading for the long stem thermometer was about 1° higher than

TABLE III

Molecular Weight by Rast's Camphor Method

Preparation No.	Weight of sample	Weight of camphor	Temperature Δ	Molecular weight	
	mg.	*mg.*	*°C.*		
M 184..........................	0.415	3.968	14	298.8	
M 184..........................	0.218	3.960	10	294.6	
M 184..........................	0.316	3.201	13.5	292.5	
M 184..........................	0.206	3.749	7.5	298.0	
				Average, 294.7	
				Found	Theoretical
Control samples					
Carbanilide...................	0.426	3.539	22	219	212
"	0.212	2.717	15	208	212
Cholesterol..................	0.349	1.804	20	386	386
"	0.300	5.883	5.2	392	386

$$M = \frac{40 \times \text{weight of sample} \times 1000}{\Delta \times \text{weight of camphor}}.$$

TABLE IV

Melting Points

Preparation No.	Melting point, long stem thermometer, uncorrected	Melting point, standardized short stem thermometer, uncorrected	Solidified, remelted (long stem)
	°C.	*°C.*	*°C.*
M 184..........................	273		273
M 189..........................	272.3		
M 190..........................	271.8		
M 191 d.......................	272.4		
M 194..........................	273.8		
E 194 g........................	273.0	281.2	272.4
M 199..........................	273.8		
M 200 e........................	273.3		
M 201..........................	273.8		
E 194..........................	273.2	281.2	

the reading of the short stem thermometer. In two instances the tube was allowed to cool until the sample had solidified and the melting point was again taken. The agreement between the two

readings indicates melting occurred without decomposition. Slight browning occurred.

Optical Rotation

In an earlier paper values for the specific rotation of two dilute solutions were given. Since there was some question about the accuracy of these results, the determination was repeated with a more concentrated solution in 95 per cent ethyl alcohol of a preparation having a melting point of 273.8°.

$$[\alpha]_D^{n°} = \frac{+\ 0.49° \times 100}{0.4016 \times 2} = +\ 61°$$

After standing for 22 hours the rotation was unchanged.

TABLE V
Iodine Number

Preparation No.	Theelol	Iodine No.
	mg.	
M 184	10.8	85.3
M 187 d	13.7	86.2
M 190	10.6	88.5 Average, 86.7

Iodine Number

It has long been known that cholesterol uses more iodine than its one double bond accounts for. In earlier work on the follicular hormone Jordan and Ralls (unpublished) studied the determination of the iodine number of cholesterol and devised a micro procedure that gave good results with both cholesterol and other unsaturated lipids. This method has been used for cholesterol, theelin, and theelol.

The average of the values contained in Table V, 86.7, permits us to calculate a molecular weight of 292.8 if one double bond is assumed; calculated for $C_{18}H_{24}O_3$, 288.

Oxygen and Its Relation to the Hydroxyl Groups

Since our analysis of theelol showed that it contained carbon and hydrogen but no nitrogen, halogen, or sulfur, it was assumed that the difference between 100 per cent and the sum of the percentages

of carbon and hydrogen was due to oxygen. It was therefore desirable to ascertain the nature of this oxygen.

The general solubilities of the substance and its close relationship to theelin suggested the presence of hydroxyl groups. An attempt was therefore made to prepare an acetate.

The method of Peterson and West (1927) was adopted because it affords also a titrimetric method for the quantitative determination of hydroxyl groups. The acetylation of many organic compounds with acetic anhydride in pyridine proceeds smoothly and quantitatively to completion.

TABLE VI

Acetylation of Theelol

Preparation No.	Weight of sample	Blank, standard NaOH	Sample, standard NaOH	Difference in NaOH	CH₃CO per mol (titration)	CH₃CO per mol (gravimetric analysis)
	mg.	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*
M 180............................	68.8	8.935	7.76	1.175	121.8	
M 187 d.........................	45.1	8.960	8.16	0.80	126.5	
E 194............................	164.7	8.910	5.09	3.02	130.8	118.4
M 200 e.........................	154.7	8.82	5.92	2.90	133.7	117.3
Average........................					128.2	
Theoretical, 3 (OH) for C₁₈H₂₄O₃..					129	

The sample was dissolved in pyridine and acetic anhydride (3:1) and heated under a micro Hopkins reflux condenser for 21 hours at 95°. The solution was transferred quantitatively to a 250 cc. flask and ice-cold water added. The solution containing the excess acetic anhydride was titrated with standard 0.5 N NaOH in a micro burette.

Our data (Table VI) indicate the presence of three hydroxyl groups in theelol. The ester obtained by acetylating the original theelol by the Peterson and West method was filtered, dried, and weighed. The quantity of ester obtained approximated the theoretical amount.

It was necessary to isolate the ester in pure crystalline form to determine the carbon and hydrogen. The filtered and dried ester was dissolved in hot 95 per cent ethyl alcohol, the alcohol evapo-

rated, the residue dissolved in 4 cc. of pyridine, and 16 cc. of water added. A white flocculent precipitate was obtained. It was recrystallized from 90 per cent ethyl alcohol two or three times. Beautiful white crystals of the type shown in Fig. 1 were obtained. The ester was dried by heating for 3 hours *in vacuo* at 85°. The melting point was 126° (uncorrected). Molecular weight by Rast's method, 410.

Fig. 1. Triacetyl derivative of theelol (Preparation M 187 c) crystallized from 95 per cent ethyl alcohol. 32×
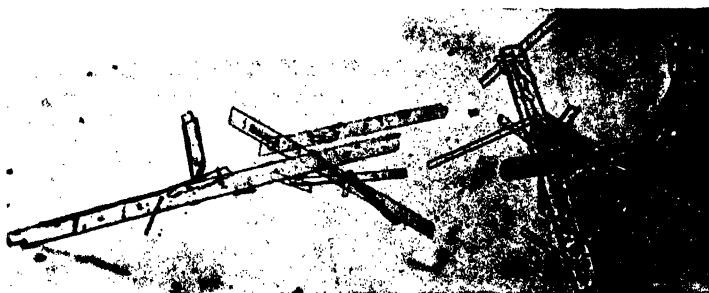
Fig. 2. Theelol (Preparation M 200 e) crystallized from 95 per cent ethyl alcohol. 120×

Hydrolysis of Triacetyl Derivative

A portion of the ester (Preparation E 194 e) was dissolved in 5 cc. of ethyl alcohol, 2 cc. of N NaOH added, and the solution heated for 3 hours and allowed to stand at 30° for 40 hours. It was diluted with 15 cc. of distilled water and acidified with HCl. The precipitate was filtered off, dissolved in ethyl alcohol, and recrystallized twice from ethyl alcohol. Microscopic examination (Preparation E 194 g) revealed the typical crystalline form (see Fig. 2) of the original theelol. Melting point 273° (uncorrected).

It seemed certain therefore that saponification of the acetate yielded the original substance again. Micro combustion analysis confirmed the view that the original substance was recovered by hydrolysis.

4.572 mg. substance (Preparation E 194 g): 12.600 mg. CO_2 and 3.480 mg. H_2O. C 75.16; H 8.5.

4.881 mg. substance: 13.410 mg. CO_2 and 3.670 mg. H_2O. C 74.93; H 8.41.

Distillation in Vacuo of the Theelol Triacetate

106.4 mg. of ester were heated in a micro distillation apparatus at a pressure of 0.026 mm. A clear viscous liquid came over at about 210° (bath temperature). The liquid did not crystallize on standing overnight but upon the addition of a few drops of ethyl

TABLE VII

Analysis of the Triacetyl Derivative

Preparation No.	Weight of sample	CO_2	H_2O	C	H
	mg.	mg.	mg.	per cent	per cent
E 194 e	4.248	10.870	2.840	69.79	7.48
E 194 e	5.087	13.000	3.380	69.70	7.43
M 200 esd	3.430	8.760	2.300	69.65	7.50
M 200 esd	3.540	9.060	2.380	69.80	7.52
Average				69.77	7.48
Theoretical for triacetyl derivative				69.53	7.30

alcohol crystals began to form. The solution was concentrated to a small volume, cooled, filtered, washed with a little ethyl alcohol, and dried. The melting point was 126° (uncorrected). The analyses given in Table VII, Sample M 200 esd, show that the distillate had the proportion of carbon and hydrogen of the triacetyl theelol.

Methylation of Theelol

A sample of theelol was treated with dimethyl sulfate in an alkaline solution and finally heated under a micro Hopkins reflux condenser for 3 hours. The precipitate was filtered off, dissolved in ethyl alcohol, and the ether precipitated with 4 volumes of 0.2 N NaOH. The precipitate was filtered off, dissolved in ethyl alcohol,

and recrystallized twice from ethyl alcohol.   Melting point 153.5°
(uncorrected).   167 mg. were distilled at a pressure of 0.02 mm. of
Hg.   A clear, colorless liquid came over at 220–230° (bath tem-
perature), leaving in the distillation tube a solid residue (m.p.
251–252°) which was faintly yellow in color.   The distillate was
redistilled and this distillate recrystallized from alcohol by the
addition of dilute NaOH.   The white crystals were dissolved in
alcohol and precipitated with water.   After drying *in vacuo* at
110°, the melting point was 154.8° (uncorrected).

Analysis
Weight of sample 1.460 mg.: H_2O, 1.160 mg.; CO_2, 4.040 mg.
                3.010 "   2.385 "   "   8.315 "
                3.380 "   AgI, 2.640 "
For $C_{18}H_{23}O_2$ OCH_3. Theory. H 8.67,   C 75.45, OCH_3 10.27
                Found.   " 8.89, 8.86, " 75.47, 75.34,   "   10.32
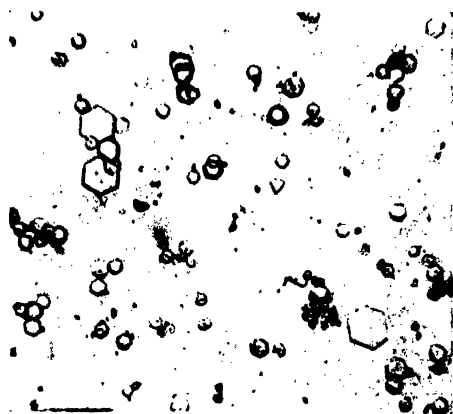
Fig. 3. Monomethyl ether of theelol; recrystallized from dilute ethyl
alcohol. 120×

The analytical data indicate clearly that the derivative is a
monomethyl ether of theelol (Fig. 3).

DISCUSSION

Our physiological evidence indicates that two different estrogenic
substances are present in the extracts of pregnancy urine and we
believe that our chemical and physiological evidence warrants the

stand that we have isolated those two compounds in a chemically
pure condition.

Theelol has been isolated as a colorless crystalline compound with
a melting point of 273–274°. Specimens have been acetylated
giving a triacetyl derivative which has been purified by two differ-
ent procedures; in one instance it was recrystallized and in the other
it was recrystallized and then distilled *in vacuo.* Both products
had the same melting point (126°). The ester was then hydrolyzed
and the original triol recovered with its melting point and per cent
of carbon and hydrogen unchanged. Bioassay of the original
Preparation E 194 and Sample E 194 g recovered after hydrolysis
of the ester gave the same number of rat units per mg.

Our data lead to the belief that both theelin and theelol are prob-
ably derivatives of the same unsaturated hydrocarbon. The·
number of carbon atoms per molecule is the same but theelol con-
tains 2 atoms of hydrogen and 1 atom of oxygen more than theelin.
This indicates the addition of a hydroxyl and the reduction of the
carbonyl of theelin to a hydroxyl group. No doubt double bonds
exist because the saturated hydrocarbon with 18 carbons would
contain 38 atoms of hydrogen. In theelol, however, we find 24
atoms of hydrogen giving a probability of seven double bonds, if
we exclude ring structures, of which only one adds iodine by the
Jordan-Ralls procedure. Since only one double bond adds iodine
in both theelol and theelin one might hazard a guess that the other
unsaturations occur in a benzene nucleus. It seems rather appar-
ent that further work is necessary to decide the structures of
theelol and theelin and the chemical relationship between them.

Admitting that a relationship exists between theelol and theelin,
it becomes important to decide which is the follicular hormone.
Both, one, or neither may be secreted by the ovary and placenta.
The substances isolated from the urine may have come from prod-
ucts which have served their functions in the body and in so doing
have been altered and are then eliminated as waste products in
the urine. Or, we might suppose that one or the other is the
follicular hormone and that the other is formed by the process of
extraction and purification. It seems to the authors that this is
the more probable view and furthermore that of the possible trans-
formations, the change from theelin to theelol is the more probable.
We believe that it would be easier to introduce than to remove the

hydroxyl group. The treatment with hot alkali might be regarded with suspicion.

SUMMARY

1. Theelol has been characterized as a compound composed of carbon, hydrogen, and oxygen in such proportions as are expressed by the formula $C_{18}H_{24}O_3$. Molecular weight determination confirmed this formula.

2. That the oxygen atoms exist as hydroxyls is shown by the preparation of a triacetyl compound having a melting point of 126°. The original triol has been regenerated by hydrolysis.

3. The monomethyl ether having a melting point of 154.8° has been prepared. The methylation appears to have occurred at the hydroxyl responsible for the solubility of the triol in aqueous alkali.

We are glad to record our indebtedness to Dr. O. Wintersteiner for personal instruction to Dr. Thayer in the use of microanalytical methods.

Addendum—In a recent paper, Butenandt (*Abhandl. Ges. Wissensch. Göttingen, Math.-physik. Klasse, 3*, pt. 2 (1931)) reports that he has isolated the triol discussed in this paper. One of his preparations melted at 273° with browning and decomposition; the others at 268–269°. The compound was analyzed for carbon and hydrogen and found to contain those elements in the proportion demanded by the formula $C_{18}H_{24}O_3$. By heating to 180–200° with fused potassium bisulfate, sublimation of progynon ($C_{18}H_{22}O_2$, theelin) was effected.

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CRYSTALLOGRAPHIC DESCRIPTION OF THEELOL

By CHESTER B. SLAWSON

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For the following determinations two separate samples of theelol were used, one of which had been recrystallized from 25 per cent and the other from 95 per cent ethyl alcohol. No variation in the optical properties between these two samples was observed, although there was a slight variation in the external form of the crystals. Theelol recrystallized from 25 per cent alcohol is better adapted to micro optical determinations so the diagrams show the type of crystal formed from such a solution. The crystals (Preparation E 194) prepared from a 95 per cent alcohol solution are elongated parallel to the vertical axis[1] and in some cases the acute angles shown in Fig. 1 are truncated by small crystal faces.

The crystals are monoclinic forming rectangular parallelopipeds. Fig. 1 shows the side view of these crystals and Fig. 2 the front view. These are the characteristic outlines observed under the microscope because the crystals tend to lie in either one or the other of these positions upon the slide.

The crystals are optically negative; the indices of refraction are $\alpha = 1.533$, $\beta = 1.642$, and $\gamma = 1.686$ (all ± 0.003); and $2V$ is approximately $60°$. The obtuse bisectrix is perpendicular to the side pinacoid and therefore α and β may be measured upon all crystals lying in such a position as to give the rhomboid outline shown in Fig. 1. The extinction angle is $5\frac{1}{2}°$, measured from the longer edge of the rhomboid in the direction of the obtuse angle. α is measured parallel to this direction and β perpendicular to it. Crystals lying in such a position as to give the rectangular outline shown in Fig. 2 have parallel extinction and α may be measured parallel to the longer edge and γ parallel to the shorter edge. A

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[1] See Fig. 2 in the paper by Thayer, S. A., Levin, L., and Doisy, E. A., *J. Biol. Chem.*, **91**, 655 (1931).

distinct basal cleavage was observed. Although the crystals are very small they are extremely well developed and give sharp and distinct outlines under the microscope. Because of the tendency of the crystals to lie in two characteristic positions upon the slide, no difficulty is encountered in measuring the three indices of refraction.
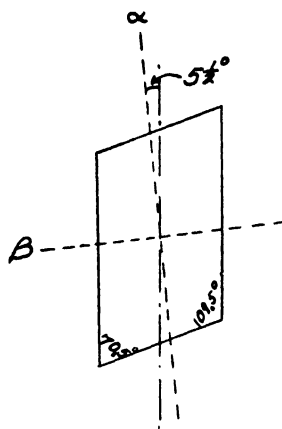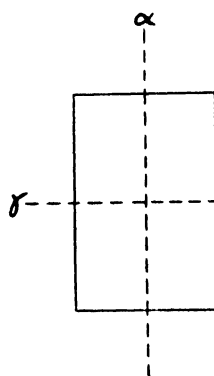
FIG. 1   FIG. 2

FIGS. 1 AND 2. Side and front views of the theelol crystal

TABLE I

Comparison of Theelin and Theelol Crystallized from 25 Per Cent Ethyl Alcohol

	Inter-facial angle	Indices of refraction			2V	Extinc-tion angle
		α	β	γ		
	degrees				*degrees*	*degrees*
Theelol......................	$70\frac{1}{2}$	1.533	1.642	1.686	60	$5\frac{1}{2}$
Theelin......................	68	1.520	1.642	1.692	55	12

Theelol bears a very close relationship to theelin in all its crystallographic properties (Slawson, 1930)[2] as Table I shows.

The maximum experimental error in these values should not be greater than 1° in the angular values and 0.003 for the indices of

[2] Slawson, C. B., *J. Biol. Chem.*, **87**, 373 (1930).

refraction. In the above determinations the values for the α and β indices of refraction are probably accurate to 0.001.

For the purpose of differentiating the two compounds a determination of either the α index of refraction or the extinction angle should be satisfactory. The best immersion liquids to use are mixtures of paraffin oil and α-monobromonaphthalene because both compounds are insoluble in these liquids.

ARE THE WILLIAMS-WATERMAN VITAMIN B₃ AND RANDOIN-LECOQ NUTRITIONAL VITAMIN THE SAME?

By RAOUL LECOQ

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The complex nature of the original vitamin B is well recognized today, opinions differing only as to the number of fractions entering into its constitution.

The antineuritic factor, first extracted by Funk (1), is adsorbed by fullers' earth. This vitamin is variously designated as vitamin B, B₁, or F.

On the other hand, the work of Smith and Hendrick (2) and of Goldberger and collaborators (3) has shown that there also exists a thermostable, antipellagrous factor variously designated as vitamin B₂, P-P, or G. This factor, as found by Hassan and Drummond (4), resists autoclaving in an alkaline medium. These two distinctive characteristics, thermostability and stability in alkaline medium, are exactly those attributed by Funk and Dubin (5) to their yeast growth-promoting factor, or vitamin D.

Randoin and Lecoq (6) in 1926, by means of brewers' and distillers' yeasts, showed that pigeons must receive, in addition to the antineuritic vitamin, a growth-promoting substance or nutritional factor. This they called "vitamine d'entretien ou de fonctionnement" or "vitamine d'utilisation nutritive." At this time, Randoin and Lecoq also showed that certain yeasts or yeast extracts prevented loss of weight but not polyneuritis in adult pigeons, a fact independently confirmed soon after by Hauge and Carrick (7). It was also shown by Randoin and Lecoq that yeast extracts autoclaved in alkaline medium were not sufficient to maintain the weight of pigeons, and they then admitted provisionally that this yeast fraction was vitamin-free. However, Funk and Lecoq (8) pointed out in this yeast extract fraction, as well

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as in Harris' extract, the presence of the yeast growth-promoting vitamin. Goldberger and coworkers (3) had shown the Harris extract to be antipellagrous.

Thus, in 1927, Lecoq (9) admitted the necessity of supplying pigeons with three or four distinct vitamins B, depending upon whether one accepts as identical or not the antipellagrous factor and the yeast growth-promoting factor of Funk and Dubin. This latter factor (antipellagrous or yeast growth-promoting), together with the antineuritic and the nutritional factor, was present in the original experiments of Randoin and Lecoq (6).

This finding, though in disagreement with the work of Seidell (10), was later corroborated by our own (11), in which it was shown that the thermostable and alkali-stable fraction of yeast (vitamin B_2) is undoubtedly required by pigeons. This is insufficiently supplied by the activated fullers' earth fraction when prepared in alcoholic medium and which is strictly antineuritic.

Thus it seems that for pigeons, in addition to the factors B_1 and B_2, a third, the "nutritional vitamin" of Randoin and Lecoq is also necessary. However, Williams and Waterman (12) in 1927 pointed out that in addition to vitamins B_1 and B_2 the pigeon requires a third vitamin B factor, distinct from these two, which they designated as B_3.

Are the Williams-Waterman vitamin B_3 and "nutritional vitamin" of Randoin and Lecoq identical? According to the latter investigators, the "nutritional vitamin" (a good source of which is malt extract (13)) is destroyed by autoclaving in an alkaline medium, is less readily adsorbed by fullers' earth, and is more resistant to heat than the antineuritic factor. The Williams-Waterman vitamin B_3 appears more thermostable than the antineuritic vitamin. Furthermore, Eddy, Gurin, and Keresztesy (14) have shown that malt extracts made at temperatures as low as 60° are practically devoid of vitamin B_3, though still very effective as sources of vitamin B_1. On the other hand, the destruction of the yeast antineuritic vitamin observed by Randoin and Lecoq (15) in drying distillers' yeast appears to be contrary to the more rapid loss of vitamin B_3 in the drying of brewers' yeast as observed by Eddy and collaborators.

But it seems that we can attribute these discrepancies to the different origin of the materials used and to the action of diastases

which are present and which unquestionably play a large part in preventing the destruction of these factors. In this connection, Lecoq (16) pointed out the absence of amylase in dry French malt extract, and it is perhaps to such an absence that the preservation of the "nutritional vitamin" is due.

We must, of course, be cautious in comparing experiments that are not conducted in the same way, especially in regard to the origin of the yeast and the nature and proportion of the carbohydrates used (17). Further, the forced feeding employed in the French experiments is in our opinion an advantage but is also apt to involve difficulty in comparing results.

It appears, however, from the considerations set forth above, that the similarities between the two vitamins, the Williams-Waterman B₃ and our "nutritional vitamin," exceed their discrepancies and that they are the same.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

IV. CALCIUM AND PHOSPHORUS UTILIZATION IN LATE LACTATION AND DURING SUBSEQUENT REPRODUCTIVE REST*

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In women the physiological processes motivated by lactation
are accompanied by increased requirements for minerals (1) and
other food essentials (2). The finding of significant losses of
calcium during the period of intensive milk flow, despite a calcium
intake seemingly adequate to maintain bodily function with a
rich surplus to accommodate the added demands of milk secretion,
and the addition of vitamin supplements to the dietary (3), points
to the need for a more thorough knowledge of the physiological
processes involved in reproduction and of the applicability of the
science of nutrition to the conservation of the maternal body for
a better fulfilment of its supreme function—the bearing and nurturing of strong, healthy children.

The nature and far-reaching effects of intensive and protracted
calcium losses during any phase of the reproductive cycle of women
(4) are unknown. A progression of pregnancies, followed by long
and copious lactation with little or no reproductive rest interven-

ing, may cause exhaustive drains upon the maternal body. It has been shown that in the case of two women there was a storage of calcium and phosphorus in the later part of the second and third lactation periods, respectively, when there was a progressively decreased output of milk; whereas in the succeeding lactation periods of these women, in which there was a continuous, more intense output of milk, negative calcium balances persisted. The factors that may influence calcium utilization have been reviewed by Coons and Blunt (5) and by the staff of these laboratories (1, 3, 6).

In order to obtain further information concerning mineral metabolism under varying conditions during the reproductive cycle, studies of the calcium and phosphorus balances of three women accustomed to producing large quantities of milk were made at the close of lactation periods during the progress of which each of them had experienced continued calcium losses and at intervals during the subsequent postlactation period in two of the women.

Procedure

Three American women who have been for several years the subjects of investigations on the metabolism of women during the reproductive cycle were observed for the following report. The average daily milk output for the lactation periods under observation was for Subject VI, 3134 cc.; for Subject VII, 2366 cc.; and for Subject VIII, 1419 cc. Milk in excess of the demands of the infants was sold by the mothers to the Mother's Milk Bureau of Detroit. Milk secretion was maintained over a period of 14 months. The metabolic observations were conducted in the homes of the subjects. The procedures and methods followed in the management of the studies have been described in this *journal* (1, 3, 6). In another publication (7) the interpretation and evaluation of the results of human metabolic balances, including a comparison of the short and long time observation period, are considered.

Experimental Results

At the close (60th week) of her third lactation period, Subject VI was storing both calcium and phosphorus. The output of

milk had progressively decreased during the last half of lactation
(Chart I). At the cessation of milk flow, the fourth pregnancy
followed immediately. It was terminated with a full term infant
weighing 8 pounds 10 ounces. In the fourth lactation cycle a
loss of calcium was experienced in the 7th week post partum, the
loss had markedly increased in the 27th week. Thereafter the
ingestion of 15 gm. of cod liver oil and 10 gm. of yeast daily over
a 2 months period stimulated better utilization of calcium, with a
consequent storage (3). 6 months after the discontinuance of
these adjuvants, with maximal milk flow continuing, there was
again a loss of calcium. At the 63rd week of lactation a short time
balance determination indicated that Subject VI was losing cal-
cium (Chart II) equivalent to 0.80 gm. per day. The intake of
2.96 gm. daily in the fourth lactation was virtually the same as
that in a similar period of the third lactation, but 0.28 gm. more
calcium was secreted in the milk in the fourth lactation (Chart II).
As shown in Table I, the phosphorus metabolism was quite similar
at the close of the third and fourth cycles in magnitude of intake,
in the outgo through the several paths of excretion, and in the
amount stored (Chart III). Though she had experienced one
less pregnancy and lactation than Subject VI, Subject VII mani-
fested much the same status of mineral metabolism at similar
intervals of the lactations under observation. Her third preg-
nancy followed immediately after the termination of the second
lactation. A full term infant was born which weighed 9 pounds
and 3 ounces. She was storing calcium at the end of the second
lactation period, but losses were noted in the early and middle
periods of the third lactation. Although the administration of
cod liver oil and yeast did not stimulate calcium storage, they
served to diminish the. negative balance (Table I and Chart II).
In the 62nd week of the third lactation, just preceding cessation
of mammary function, Subject VII was losing calcium (1.16 gm.
per day); the intake was 2.11 gm. and the outgo in the milk 0.46
gm. There was a daily storage of 0.27 gm. of phosphorus (Chart
III), which was less than at the 50th week of her second lactation.
In the third lactation the demand for minerals was both greater
in magnitude and sustained for a longer length of time than in
the second lactation. The average daily milk output was 366 cc.
greater and the period was 12 weeks longer.

OUTPUT OF MILK
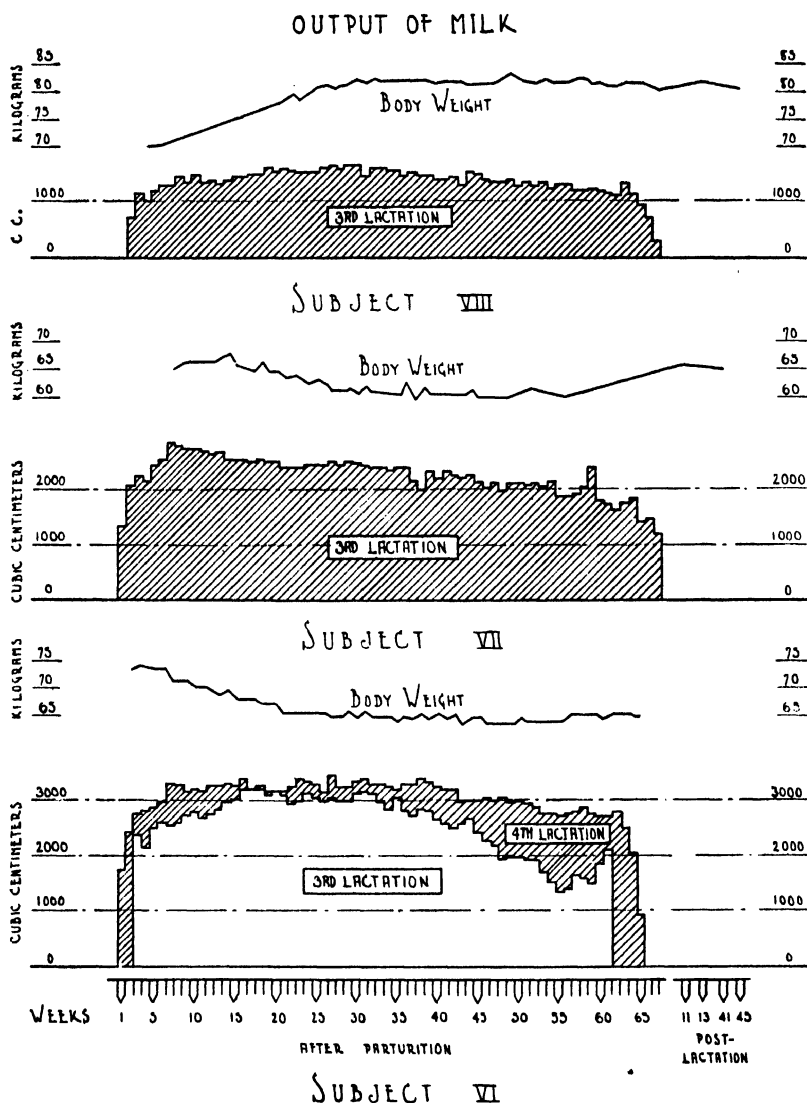
CHART I. Average daily milk output in weekly intervals and weekly body weights for three women. Two successive lactation periods, the latter being much more intense, are given for Subject VI in her third and fourth periods and one for each Subject VII and Subject VIII. The entire milk supply was expressed by hand two to four times daily and quantitative measurements recorded.

CALCIUM BALANCES
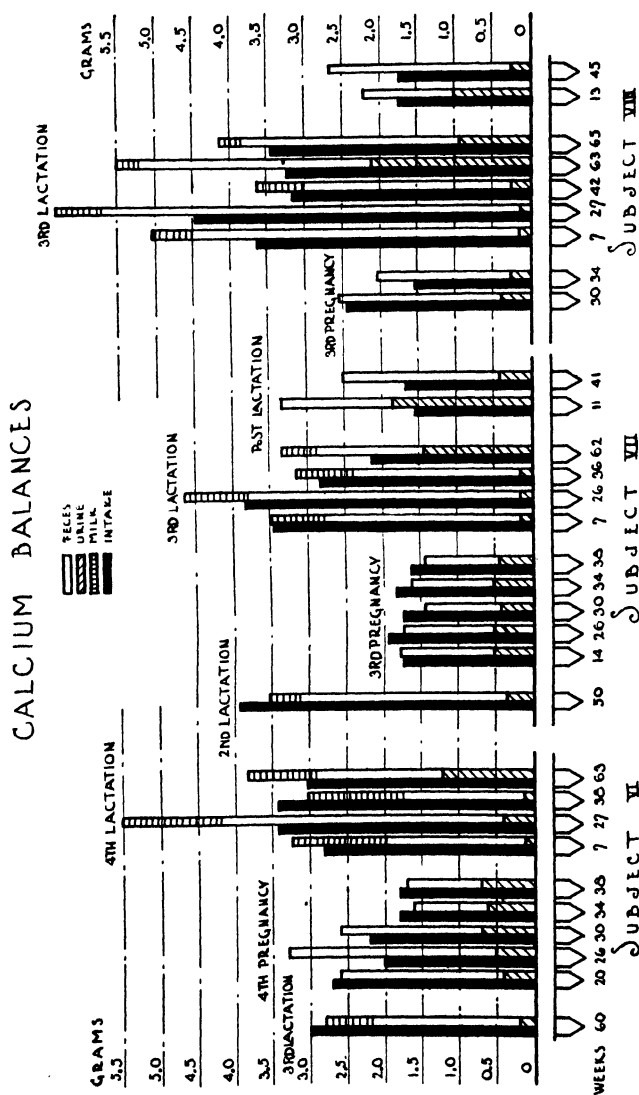
CHART II. Daily calcium exchange in pregnancy, lactation, and postlactation. The solid black columns illustrate the total number of gm. of calcium in the daily food intake; the plain and shaded columns show the total outgo of calcium in the feces, urine, and milk. The difference between the height of the intake and outgo columns represents the calcium balance. When the solid column exceeds the outgo, calcium is being stored in the body; on the other hand, when the opposite condition exists, calcium is being lost.

CHART III. Daily phosphorus exchange in pregnancy, lactation, and postlactation. The solid black columns illustrate the total number of gm. of phosphorus in the daily food intake; the plain and shaded columns show the total outgo of phosphorus in the feces, urine, and milk. The difference between the height of the intake and outgo columns represents the phosphorus balance. When the solid column exceeds the outgo, phosphorus is being stored in the body; on the other hand, when the opposite condition exists, phosphorus is being lost.

The demands of continuous reproduction in women no doubt exhaust the labile sources of calcium (8) and mobilize and transport some of the more stable depots. It would seem pertinent to know how long it takes the maternal organism to readjust itself after the conclusion of the augmented physiological demands of maternity. Metabolic balance studies in postlactation should yield information upon the subject. 11 weeks after cessation of lactation in Subject VII, a metabolism study showed a calcium loss of 1.79 gm. daily and the phosphorus metabolism in a state of equilibrium. Furthermore, 41 weeks after cessation of mammary activity, when it was assumed that the maternal organism could have adjusted itself after the period of milk secretion and reproductive drain, a further loss of calcium was suffered. During a balance period of 9 days in length there was a loss of 0.75 gm. of calcium per day while on the other hand there was a storage of 0.22 gm. of phosphorus. The mineral intake was maintained approximately at the same level as at the 11th week postlactation.

Metabolic studies were conducted on Subject VIII at intervals throughout her third reproductive cycle and during subsequent rest. The infant weighed 6 pounds, 12 ounces. During early and mid-lactation Subject VIII experienced a greater drain of calcium than did either Subject VI or VII, although her intake was greater and her milk output approximately 50 per cent less. Cod liver oil and yeast stimulated better utilization of calcium, but the loss nevertheless persisted and was accentuated after the withdrawal of these dietary supplements. Subject VIII was observed at the 63rd and 65th weeks of the third lactation period. At the 63rd week the output of milk was maintained at 1323 cc., an amount only slightly lower than the average output for the entire lactation cycle (Chart I). In the following 2 weeks, the milk volume was voluntarily decreased to about 952 cc., by reducing the daily number of times of emptying the breasts from four to one or two. The calcium intake for both periods was practically the same (approximately 3 gm.), but the outgo in the milk was 0.08 gm. less at the 65th week (Chart II). At the 63rd week a daily loss of 2.32 gm. of calcium was indicated; at the 65th week a loss of 0.66 gm. The maternal body was found to be storing phosphorus (Chart III) during both periods; the intake was 3.74 gm. at the 63rd week and 3.22 gm. at the 65th week.

TABLE I

Calcium and Phosphorus Metabolic Balances

Subject No.	Subject history	Period	Week postpartum	Length of period	Average daily milk output	Calcium						Phosphorus					
						Intake	Milk	Urine	Feces	Total	Metabolic balance	Intake	Milk	Urine	Feces	Total	Metabolic balance
				days	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
VI	Tripara Age, 25 yrs.; height, 62.2 in.; weight, 63.1 kilos	Third lactation (A)	60	10	1923	2.97	0.58	0.20	1.99	2.77	+0.20	3.27	0.25	1.62	1.01	2.88	+0.39
	Quartipara Age, 27 yrs.; weight, 65.5 kilos†	Fourth lactation (B)	7	4	3213	2.82	1.24	0.08	1.92	3.24	−0.42	2.98	0.48	1.82	0.79	3.09	−0.11
			27	4	3448	3.39	1.30	0.40	3.85	5.55	−2.16	3.61	0.40	2.79	1.23	4.42	−0.81
			38‡	4	3417	3.41	1.17	0.16	1.66	2.99	+0.42	3.62	0.37	1.35	0.48	2.20	+1.42
			63	4	2856	2.96	0.86	0.23	1.67	3.76	−0.80	3.05	0.35	1.59	0.85	2.79	+0.26
VII	Duopara Age, 33 yrs.; height, 68.5 in.; weight, 64.5 kilos	Second lactation (A)	50	10	1197	3.92	0.28	0.32	2.83	3.43	+0.49	3.33	0.20	1.78	1.56	3.54	+0.79
	Tripara Age, 35 yrs.; weight, 61.8 kilos†	Third lactation (B)	7	4	2683	3.36	0.71	0.07	2.64	3.42	−0.06	3.14	0.47	1.36	1.50	3.33	−0.19
			26	4	2458	3.83	0.84	0.07	3.67	4.58	−0.75	3.58	0.38	0.89	1.69	2.96	+0.62
			36‡	4	2506	2.76	0.83	0.13	2.18	3.14	−0.38	3.31	0.38	0.95	1.18	2.51	+0.80
			62	3	1739	2.11	0.46	1.36	1.45	3.27	−1.16	2.84	0.30	1.17	1.10	2.57	+0.27

Weight, 66.8 kilos	Postlactation	11	3		1.48	1.82		1.45	3.27	−1.79	2.08		0.95	0.96	1.91	+0.17
Weight, 65.9 kilos		41	9		1.73	0.41		2.08	2.49	−0.76	1.86		0.74	0.90	1.64	+0.22
VIII Tripara — Age, 29 yrs.; height, 62.6 in.; weight, 81.8 kilos†	Third lactation (A)	7	4	1490	3.62	0.47	0.13	4.37	4.97	−1.35	3.24	0.26	1.82	1.19	3.27	−0.03
		27	4	1654	4.42	0.53	0.13	5.61	6.27	−1.85	3.22	0.23	0.47	1.27	1.97	+1.25
		42‡	4	1518	3.10	0.45	0.15	3.06	3.66	−0.56	3.62	0.19	1.38	0.87	2.44	+1.18
		63	4	1323	3.18	0.35	2.05	3.10	5.50	−2.32	3.74	0.17	1.75	1.59	3.51	+0.23
		65	3	952	3.39	0.27	0.89	2.89	4.05	−0.66	3.22	0.11	1.55	1.39	3.05	+0.17
Weight, 81.4 kilos	Postlactation	13	4		1.68	1.01		1.20	2.21	−0.53	1.85		1.03	0.58	1.61	+0.24
Weight, 80.9 kilos		45	9		1.68	0.23		2.36	2.59	−0.91	1.91		0.90	0.71	1.61	+0.30

* In postlactation periods; weeks after cessation of lactation.
† End of lactation.
‡ Cod liver oil and yeast period.

Observations similar to those on Subject VII were made on Subject VIII at the 13th and 45th weeks after cessation of lactation.   At the 13th week, when the intake of calcium was 1.68 gm., there was a loss of 0.53 gm.; at the 45th week, when a balance period of 9 days comprised the observation time, the same intake as of the former period was maintained with a loss of 0.91 gm. daily for the entire period.   The results show a retention of 0.24 gm. of phosphorus at the 13th week postlactation with an intake at 1.85 gm. and a retention of 0.30 gm. at the 45th week with an intake of 1.91 gm.

<div align="center">DISCUSSION</div>

Two women, Subjects VI and VII, when observed at the close of the fourth and third lactation periods, respectively, were losing calcium, in contrast to a storage of this element at the close of their third and second lactation periods.   The intake of calcium was copious (2 to 4 gm. daily) and from a variety of sources, chief of which was milk; therefore the losses determined cannot be attributed to a meager milk consumption.   The cumulative effect of progressive demands imposed on the maternal organism by the preceding pregnancies and subsequent periods of milk flow, following in very close succession may have had a bearing upon the negative calcium balance.

The differences in calcium metabolism at the close of two successive lactation cycles in the same women may be explained partially through the milk production curves.   By superimposing the mature milk production curve of the third lactation period of Subject VI on that of her fourth period (Chart I), it can be noted that the latter was longer in duration and greater in magnitude than the former.   The diminution in output of milk began much earlier in the third lactation period; in the fourth period a higher quantity output was maintained until about 8 weeks before the cessation of milk flow.   In the later part of her second lactation period, when milk production progressively decreased, Subject VII experienced a storage of calcium and phosphorus in late lactation.   These findings are in accord with those of investigators in the dairy industry (9–11).   The curves of Subjects VII and VIII (Chart I) in their third lactation periods are characterized by a continuous high quantity production of milk throughout the

entire lactation cycle. Continuous maximum milk production over 14 months led to negative calcium balances in the three women.

It is of interest to note that with but few exceptions the phosphorus balances throughout the reproductive cycle have been positive, a finding which is in contrast to a loss of this element observed in rats (12) during lactation. The significance of this continued positive balance of phosphorus has yet to be explained. At a later time the phosphorus metabolism will be considered in relation to the nitrogen metabolism, which was observed simultaneously by short time metabolic periods at chosen intervals.

12 months after the cessation of mammary function, calcium losses and phosphorus storage continued. From these results it seems that the maternal organism requires many months to readjust itself after closely repeated pregnancies and long, intense lactations. Such findings demonstrate a real need for some means of accelerating the return of calcium metabolism to a plane of equilibrium or storage in mothers who have suffered a depletion during the reproductive cycle.

CONCLUSION

Calcium balance determinations for three women during the later part of a lactation period in which there had been long continued, intense milk flow, indicate a loss of calcium, in contrast to a storage of the element at corresponding periods in the preceding lactation period of each woman, in which there was a long, progressive decline in the output of milk. There was a storage of phosphorus in all cases at the close of 14 months of lactation. During the postlactation periods, at the 3rd and 12th months after cessation of milk flow, calcium losses and phosphorus storage were indicated.   .

Addendum—Since this manuscript was written the work of Toverud and Toverud (13) has been made available to us. Their work indicated that lactating women may store or lose calcium and phosphorus up to 5½ months of lactation.

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ON WALDEN INVERSION

XVI. THE INFLUENCE OF SUBSTITUTING GROUPS ON OPTICAL ROTATION IN THE SERIES OF DISUBSTITUTED PROPIONIC ACIDS CONTAINING AN ETHYL GROUP

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The primary object of the present investigation was to obtain further data on the effect on the optical rotation of the substitution of a hydroxyl by a halogen. Incidentally, it was hoped to obtain additional data which might lead to a deeper understanding of the relationship between chemical structure and optical activity.

Three series of substances have now been studied in which the substitution is accomplished definitely without Walden inversion inasmuch as in these substances the group to be substituted is allocated not directly on the asymmetric carbon atom. The differences in the structures of the substances in the three series can be seen from the following figures, which represent the three parent substances

$$
\begin{array}{ccc}
CH_3 & CH_3 & C_2H_5 \\
| & | & | \\
HC{-}COOH & HC{-}CH_2COOH & HC{-}CH_2COOH \\
| & | & | \\
R & R & R \\
I & II & III
\end{array}
$$
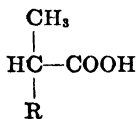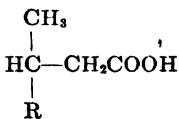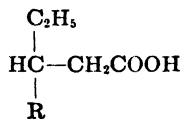
(Group R being aliphatic and higher than methyl in Series I and II and higher than ethyl in Series III).

Each member of Series I differs from that of Series II by one link, CH_2, between the polar group and the asymmetric carbon atom. Series II and III differ by the character of one of the groups attached to the asymmetric carbon atom. This group is a

methyl in Series II and an ethyl in Series III.   Thus, a comparison of the three series permits the study, on one hand, of the effect on the rotation of a given polar group as a function of its distance from the asymmetric carbon atom, and on the other hand, of the respective effects on the rotation of the groups

$$CH_3 \qquad\qquad C_2H_5$$
$$|\qquad\qquad\qquad |$$
$$HC— \quad \text{and} \quad HC—$$
$$|\qquad\qquad\qquad |$$
$$R \qquad\qquad\qquad R$$
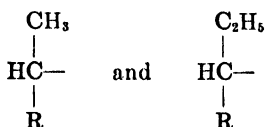
Configurational Relationship of the Substances of the Three Series—Before the effect of substitution on the rotation can be studied intelligently, it is necessary first to correlate the configurations of the members of each series among themselves and second, to correlate the configurations of the series among each other.

Series I—In the series of aliphatic disubstituted acetic acids all members on substitution of one polar group by another behave similarly.   The parent-substituted acetic acids rotate in the same direction and, therefore, there was no reason to suspect any configurational differences between the individual disubstituted acetic acids (the substituting groups being aliphatic) rotating in the same direction.

Series II—In this series the first member, namely 1,1-methylethylpropionic acid (3), and the higher members were configurationally related when they rotated in opposite directions.   Evidence to this effect was produced in a previous paper by Levene and Marker.[1] Additional evidence is furnished in this communication; namely, it is now shown that dextro-methylethyl carbinol leads to dextro-1,1-methylethylpropionic acid (3) whereas the configurationally related dextro-methylpropyl carbinol leads to the levo-1,1-methylpropylpropionic acid (3) (see the accompanying set of figures).

Series III—Considering 1,1-ethylpropylpropionic acid (3) as the first member of the series, it was found that the levorotatory first member of the series was configurationally related to the dextrorotatory higher members.   The evidence for this view is anal-

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).
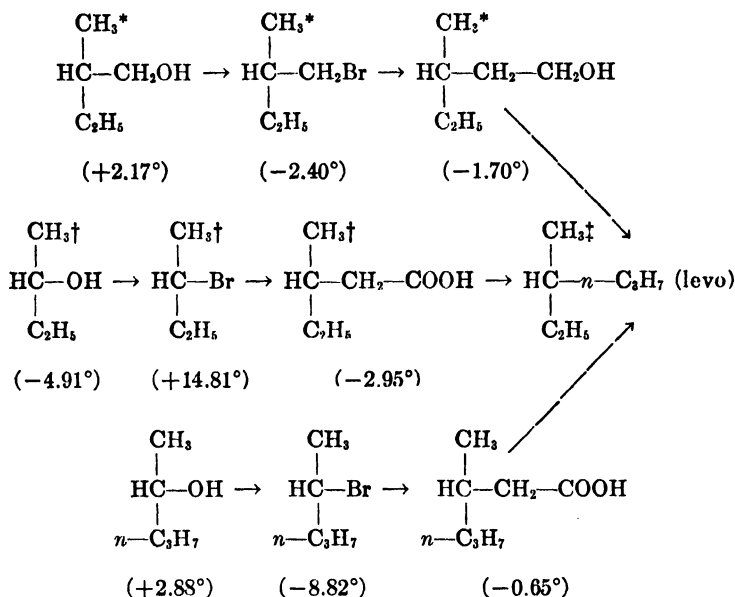
$$
\underset{\substack{|\\ \mathrm{C_2H_5}}}{\overset{\substack{\mathrm{CH_3^*}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{CH_2OH} \;\rightarrow\;
\underset{\substack{|\\ \mathrm{C_2H_5}}}{\overset{\substack{\mathrm{CH_3^*}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{CH_2Br} \;\rightarrow\;
\underset{\substack{|\\ \mathrm{C_2H_5}}}{\overset{\substack{\mathrm{CH_2^*}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{CH_2}\!-\!\mathrm{CH_2OH}
$$

$(+2.17°) \qquad (-2.40°) \qquad\qquad (-1.70°)$

$$
\underset{\substack{|\\ \mathrm{C_2H_5}}}{\overset{\substack{\mathrm{CH_3\dagger}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{OH} \;\rightarrow\;
\underset{\substack{|\\ \mathrm{C_2H_5}}}{\overset{\substack{\mathrm{CH_3\dagger}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{Br} \;\rightarrow\;
\underset{\substack{|\\ \mathrm{C_7H_5}}}{\overset{\substack{\mathrm{CH_3\dagger}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{CH_2}\!-\!\mathrm{COOH} \;\rightarrow\;
\underset{\substack{|\\ \mathrm{C_2H_5}}}{\overset{\substack{\mathrm{CH_3\ddagger}\\ |}}{\mathrm{HC}}}\!-\!n\!-\!\mathrm{C_3H_7}\ \text{(levo)}
$$

$(-4.91°) \qquad (+14.81°) \qquad (-2.95°)$

$$
\underset{\substack{|\\ n\!-\!\mathrm{C_3H_7}}}{\overset{\substack{\mathrm{CH_3}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{OH} \;\rightarrow\;
\underset{\substack{|\\ n\!-\!\mathrm{C_3H_7}}}{\overset{\substack{\mathrm{CH_3}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{Br} \;\rightarrow\;
\underset{\substack{|\\ n\!-\!\mathrm{C_3H_7}}}{\overset{\substack{\mathrm{CH_3}\\ |}}{\mathrm{HC}}}\!-\!\mathrm{CH_2}\!-\!\mathrm{COOH}
$$

$(+2.88°) \qquad (-8.82°) \qquad\quad (-0.65°)$

* For convenience of discussion the signs of rotation of all members of this series were changed from those found experimentally. All the values are in $[\mathrm{M}]_D^{23\,\pm\,2°}$ (in degrees).

† Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405 (1931).

‡ For intermediate steps from acids or carbinols to hydrocarbons see Levene and Marker.[1]

ogous to that which establishes the configurational relationship of the first and of the higher members of Series II. Namely, it was shown that dextro-1,1-ethylpropylpropionic acid (3) and dextro-1,1-ethylamylpropionic acid (3) lead to the same dextrorotatory ethylpropylamyl methane, as can be seen from the following figures.

$$
\underset{\substack{|\\ \mathrm{C_3H_7}}}{\overset{\substack{\mathrm{H}\\ |}}{\mathrm{C_2H_5C}}}\!-\!\mathrm{CH_2COOH} \;\rightarrow\;
\underset{\substack{|\\ \mathrm{C_3H_7}}}{\overset{\substack{\mathrm{H}\\ |}}{\mathrm{C_2H_5C}}}\!-\!\mathrm{C_5H_{11}} \;\leftarrow\;
\mathrm{COOH}\cdot\mathrm{CH_2}\!-\!\underset{\substack{|\\ \mathrm{C_3H_7}}}{\overset{\substack{\mathrm{H}\\ |}}{\mathrm{C}}}\!-\!\mathrm{C_5H_{11}}
$$

Dextro   Dextro   Dextro
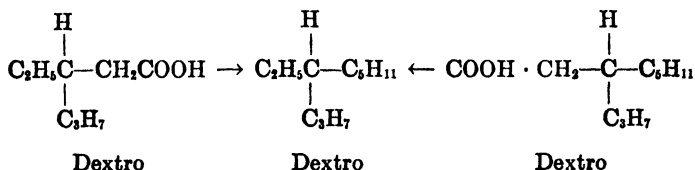
The second method was not quite so successful in this series for the reason that the reaction on condensation of dextro-ethylpropyl

TABLE I

$[M]_D^{18}$ (in Degrees)

	$-OH$	$-Br$	$-CH_2COOH$	$-CH_2COOC_2H_5$	$-CH_2CH_2OH$	$-CH_2CH_2Br$	$-CH_2CH_2Cl$	$-CH_2CHOHCHOHC_2H_5$	$-n\text{-}C_5H_{11}$	$-CH_2CH_2CH_2OH$	$-n\text{-}C_3H_7$
CH_3—HC—C_2H_5	+4.91	−14.81	+2.95 / +4.21	+4.69	+3.69		+8.95				
C_2H_5—HC—$n\text{-}C_3H_7$	+1.70	−3.35	+2.67 / Racemized	+1.02	+0.64	+1.97	+1.68	+0.56	+1.09		
$C_2H_5{}^*$—HC—$n\text{-}C_4H_9$	−8.56	+16.38	+2.13	+1.04	+0.95	+0.66					
$C_2H_5{}^*$—HC—$n\text{-}C_5H_{11}$	−8.87	+21.32	+2.87	+1.53	+1.82	+1.35 / +1.52				0	+0.76

* For convenience of discussion, the signs of rotation of all members of this series were changed from those found experimentally.

carbinol with malonic ester was accompanied by racemization. On the other hand, the dextro-ethylbutyl and dextro-ethylamylbromomethanes led to levo-1,1-disubstituted propionic acid (3), resembling in this respect the behavior of the corresponding members of Series II (see Table I).

Configurational relationships between the series of disubstituted acetic and 1,1-disubstituted propionic acids (3) have been established by the fact that the dextrorotatory methylethylpropylmethane obtained by Marckwald[2] by condensation of dextro-2-ethylpropyl iodide (3) with ethyl iodide was identical with the one obtained by us from the dextrorotatory 2-ethylbutyl bromide (4). In this manner it was shown that the series of substances given in the first column is related to the series given in the second column.

The configurational relationships of the substances given in the second and the third columns may be seen from the fact that dextro-methylpropyl carbinols lead to levo-1,1-methylpropylpropionic acid (3) and the configurationally related dextro-ethylbutyl and ethylamyl carbinols lead to levorotatory disubstituted propionic acids.

Thus, the substances given in Table II are configurationally related and therefore the group of substances may serve in the analysis of the factors determining the direction and the relative values of the rotations of simple substances.

Effect of Polarities of Substituting Groups on Optical Activity— Upon comparison not only of the directions of the rotation but also the values of individual rotations, as given in the following figures, it is found that in Series I

$$
\begin{array}{ccc}
CH_3 & CH_3 & CH_3 \\
\diagdown CHCOOH & \diagdown CHCH_2Br & \diagdown CHCH_2OH \\
C_3H_6 & C_2H_6 & C_2H_6 \\
-19.03° & -7.9° & +5.19°
\end{array}
$$
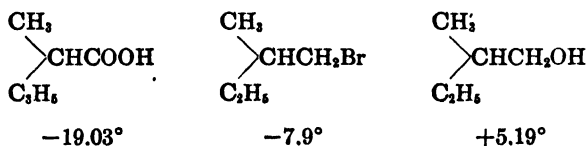
the acid has the highest levorotation, the bromide has a lower levorotation, and the carbinol is dextrorotatory. Thus with respect to this influence on rotation, the substituting groups can be arranged in the following order $COOH < CH_2Br < CH_2OH$ according to the increasing dextrorotation. It has been pointed

[2] Marckwald, W., *Ber. chem. Ges.*, **37**, 1046 (1904).

out in previous communications from this laboratory[3] and earlier by Rule[4] that this order of arrangement is analogous to that of their directive effect on the substitution in the benzene nucleus, or in order of their specific inductive capacities.

In the derivatives of the 1,1-disubstituted propionic acids (3), the acids possess the highest dextrorotation and the bromides are levorotatory (as in Series II) or possess the lowest dextrorotation (as in Series III) as can be seen from the following figures.

$$CH_3$$
$$\diagdown CHCH_2COOH \rightarrow$$
$$C_4H_9$$
$$+5.49°$$

$$CH_3$$
$$\diagdown CHCH_2CH_2OH \rightarrow$$
$$C_4H_9$$
$$+3.58°$$

$$CH_3$$
$$\diagdown CHCH_2CH_2Br$$
$$C_4H_9$$
$$+15.19°$$

$$C_2H_5$$
$$\diagdown CHCH_2COOH \rightarrow$$
$$C_4H_9$$
$$+2.13°$$

$$C_2H_5$$
$$\diagdown CHCH_2CH_2OH \rightarrow$$
$$C_4H_9$$
$$+0.95°$$

$$C_2H_5$$
$$\diagdown CHCH_2CH_2Br$$
$$C_4H_9$$
$$+0.66°$$
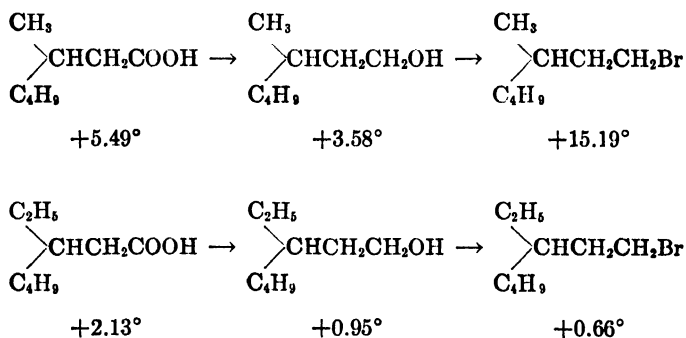
Thus, arranging the substituting group in a manner analogous to that of the arrangement of Series I, the following order is obtained. $CH_2Br < CH_2OH < COOH$. Thus in this series the acid has the highest dextrorotation whereas in the previous series the acid possessed the highest levorotation. The order $Br > OH > COOH$ is the order of the effect of the groups on the dissociation constants of substituted acids.

The significant feature of these observations, however, is the fact that whenever the polar groups are located near the asymmetric carbon atom, the halides rotate in opposite direction from the configurationally related carbinols (as in Series I and II) or at least show a drop in the numerical value of the rotation (as in Series III) which also means a change towards the opposite direction.

If the same rule were applied for the correlation of the configurations of secondary carbinols with the corresponding halides, for instance,

[3] Levene, P A., and Mikeska, L. A., *J. Biol. Chem.*, **84, 571** (1929).
[4] Rule, H. G., *J. Chem. Soc.*, **125,** 1121 (1924).

TABLE II

Lower series (CH₃ / variable chain, with CH₂Br, CH₂OH, COOH):

$$
\begin{array}{l}
\mathrm{CH_3} \\
\ \ | \\
\mathrm{HC-} \quad \{\mathrm{CH_2Br} \ \ -7.9 \\
\ \ | \qquad\{\mathrm{CH_2OH} \ \ +5.2 \\
\mathrm{C_2H_5} \ \ \{\mathrm{COOH} \ \ -19.0
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2Br} \ \ -1.4 \\
\qquad\ \{\mathrm{CH_2OH} \ \ +1.7 \\
\mathrm{C_3H_7} \ \ \{\mathrm{COOH} \ \ -6.8
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2Br} \ \ l \\
\qquad\ \{\mathrm{CH_2OH} \ \ +1.1 \\
\mathrm{C_4H_9} \ \ \{\mathrm{COOH} \ \ -6.3
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2Br} \ \ -2.93 \\
\qquad\ \{\mathrm{CH_2OH} \ \ +5.95 \\
\mathrm{C_7H_{15}} \ \ \{\mathrm{COOH} \ \ -14.18
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2Br} \ \ -0.97 \\
\qquad\ \{\mathrm{CH_2OH} \ \ +3.43 \\
\mathrm{C_{10}H_{21}} \ \{\mathrm{COOH} \ \ -12.67
\end{array}
$$

Column 1 (CH₃):

$$
\begin{array}{l}
\mathrm{CH_3} \\
\ \ | \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ 0 \\
\ \ | \qquad\{\mathrm{CH_2CH_2OH} \ \ 0 \\
\mathrm{CH_3} \ \ \ \{\mathrm{CH_2COOH} \ \ 0
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ -9.2 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ -3.7 \\
\mathrm{C_2H_5} \ \{\mathrm{CH_2COOH} \ \ -4.1
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ -16.8 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ +1.4 \\
\mathrm{C_3H_7} \ \{\mathrm{CH_2COOH} \ \ +2.8
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ -15.2 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ +3.6 \\
\mathrm{C_4H_9} \ \{\mathrm{CH_2COOH} \ \ +5.5
\end{array}
$$

$$
\begin{array}{l}
\mathrm{CH_3} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ -13.0 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ +5.4 \\
\mathrm{C_6H_{11}} \ \{\mathrm{CH_2COOH} \ \ +7.1
\end{array}
$$

Column 2 (C₂H₅):

$$
\begin{array}{l}
\mathrm{C_2H_5} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ +9.2 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ +3.7 \\
\mathrm{CH_3} \ \ \{\mathrm{CH_2COOH} \ \ +4.1
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_2H_5} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ 0 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ 0 \\
\mathrm{C_2H_5} \ \{\mathrm{CH_2COOH} \ \ 0
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_2H_5} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ -2.0 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ -0.7 \\
\mathrm{C_3H_7} \ \{\mathrm{CH_2COOH} \ \ -2.7
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_2H_5} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ +0.7 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ +1.0 \\
\mathrm{C_4H_9} \ \{\mathrm{CH_2COOH} \ \ +2.1
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_2H_5} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ +1.5 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ +1.8 \\
\mathrm{C_5H_{11}} \ \{\mathrm{CH_2COOH} \ \ +2.9
\end{array}
$$

Column 3 (C₃H₇):

$$
\begin{array}{l}
\mathrm{C_3H_7} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ +16.8 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ -1.4 \\
\mathrm{CH_3} \ \ \{\mathrm{CH_2COOH} \ \ -2.8
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_3H_7} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ +2.0 \\
\qquad\ \{\mathrm{CH_2CHOH} \ \ +0.7 \\
\mathrm{C_2H_5} \ \{\mathrm{CH_2COOH} \ \ +2.7
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_3H_7} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ 0 \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ 0 \\
\mathrm{C_3H_7} \ \{\mathrm{CH_2COOH} \ \ 0
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_3H_7} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ l \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ :: \\
\mathrm{C_4H_9} \ \{\mathrm{CH_2COOH} \ \ ::
\end{array}
$$

$$
\begin{array}{l}
\mathrm{C_3H_7} \\
\mathrm{HC-} \quad \{\mathrm{CH_2CH_2Br} \ \ d \\
\qquad\ \{\mathrm{CH_2CH_2OH} \ \ :: \\
\mathrm{C_5H_{11}} \ \{\mathrm{CH_2COOH} \ \ ::
\end{array}
$$

methylethyl carbinol and methylethylbromomethane, then the conclu-sion should be reached that the dextro-carbinol is correlated to the levo-halide. The value of the rotation of the halide, 31.98°, being higher than that of the carbinol which is 13.87°, it follows therefore that the halide rotating to the left of the dextro-carbinol can be the levorotatory only. The same conclusion was reached by Levene and Mikeska[3] on other grounds.

Exceptional Position of the Derivatives of the 1,1-Methylethyl- and of the 1,1-Ethylpropylacetic Acids—Upon comparison of the rotations of the 1,1-methylethylacetic acid and of its derivatives (see Table II) it is seen that they differ from the rotations of the other members of the same series in two respects. First, all three substances rotate in one direction, whereas in the derivatives of the other members, two substances, the acid and the carbinol, rotate in one direction and the halide in the other. Second, the 1,1-methyl-ethylpropionic acid (3) and the two derivatives are levorotatory whereas the higher 1,1-disubstituted propionic acids are dextro-rotatory as is also the carbinol derived from it but the halide is levorotatory.

The 1,1-ethylpropylpropionic acid (3) similarly to the 1,1-methylethylpropionic acid (3) is levorotatory and the carbinol and the bromide are also levorotatory whereas all the higher acids of the series are dextrorotatory, as are the carbinols and halides.

In Table II a fourth column is introduced, the higher members of which have not yet been prepared but the directions of rotations of which may be predicted from the rotations of the members of the preceding columns.

Examining the part of Table II which contains the members of the 1,1-disubstituted propionic acids, we observe a series of squares drawn by light lines which contain the symmetric members of each series. Above these symmetric members are located substances which are enantiomorphous to some members of the preceding columns. Parallel to the symmetric line follows a line of squares enclosed in heavier lines. All the substances in these squares are levorotatory. Strictly speaking, the substances con-tained in these squares are the first members of the new series inas-much as the members above the symmetric ones are enantiomor-phous to the configurationally related group of substances. Thus

there must be a reason for the exceptional behavior of the first members. What is it?

If, roughly speaking, it be assumed that the rotation of each substance containing one asymmetric carbon atom is made up of two components, one part furnished by the radicle containing the polar group and the second by the arrangement of all the radicles attached to the asymmetric carbon, then an explanation can be found for the exceptional behavior of the first members of each series by one of two possible assumptions. First, it may be argued that the first members of each series possess the least asymmetry, as there is in them the least difference in weight of the two non-polar aliphatic radicles. On the other hand, the polar radicles remain the same in the entire series. If it is assumed that in the group of substances given in Table II the radicle containing the polar group contributes the levorotatory element and the non-polar radicle the dextrorotatory element, and if the second assumption is made that the levorotatory element remains within certain limits constant, and that the values of the non-polar radicle increase progressively from the first member, then the reason for the levorotation of the first member will become self-evident; namely, in it the numerical value of the dextrorotatory element is lower than that of the levorotatory. Furthermore, it will be easy to explain the levorotation of the halides of the higher members of the second column, the reason being that the radicle containing the halogen has the highest levorotation, as is seen from the higher levorotation of the methylethyl bromo-*n*-propylmethane, as compared with the rotations of the corresponding carbinol and the corresponding acid.

The higher members of the derivatives of the 1,1-disubstituted propionic acids (3) of the third column all show dextrorotation whereas the 1,1-ethylpropylpropionic acid and the corresponding carbinol and halides are all levorotatory. Thus again it can be concluded that the numerical value of the levorotatory element is higher than the dextrotatory in the first members and lower in the higher members. The fact that halides also are dextrorotatory may indicate that in a relative sense in this series in the halides also the numerical value of the dextrorotatory element is higher than the levorotatory. The second assumption postulates that the non-polar radicles

$$\begin{array}{ccc} \mathrm{CH_3} & & \mathrm{C_2H_5} \\ | & & | \\ \mathrm{HC-} & \text{and} & \mathrm{HC-} \\ | & & | \\ \mathrm{C_2H_5} & & \mathrm{C_3H_7} \end{array}$$
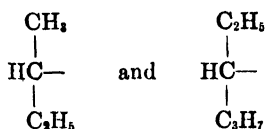
contribute a levorotation. Thus in the members enclosed in squares with heavy lines both contributions are levorotatory and hence the total rotations of the substances are levorotatory.

Respective Rotations of Members of the Disubstituted Acetic Acids and Corresponding 1,1-Disubstituted Propionic Acids (3)

Upon comparison of the rotation of the corresponding derivatives of the two series, it is observed that the acids of the corresponding members of the two series differ in the directions of their rotations. The principal difference in the structure of the substances is the presence of a CH_2 group between the carboxyl and the asymmetric carbon atom in the series of the 1,1-disubstituted propionic acids. Thus in the latter series, the carboxyl is at a greater distance from the asymmetric carbon atom and this difference in distance may be held responsible for the difference in effect on the rotation of the polar group. Similar effects of the distance on the rotation have been observed in the case of double bonds and more recently in the respective rotations of the secondary carbinols containing either an isopropyl or an isobutyl group.

SUMMARY

1. The change of rotation on substitution of the hydroxyl of the disubstituted 2,2-ethanols and of the 3,3-propanols (1) by a halogen is in the direction opposite to that of the carbinols. This observation substantiates the conclusion reached by Levene and Mikeska to the effect that dextrorotatory aliphatic secondary carbinols are configurationally related to the levorotatory secondary halides.

2. The exceptional behavior of the derivatives of 1,1-methylethylpropionic acid (3) and of 1,1-ethylpropylpropionic acid (3) is explained on the basis of the assumption that the rotation of each optically active substance may be regarded as the resultant of two components, both levorotatory, or of which one may be dextrorotatory and the other levorotatory.

3. Some radicles may function either as the dextrorotatory or the levorotatory elements depending upon their distance from the asymmetric carbon, as for instance, the carboxyl in Series I and II.

Dextro-3-Methyl-1-Pentanol—A Grignard reagent was formed from 35 gm. of magnesium in ether and 210 gm. of 1-bromo-2-methylbutane, $[\alpha]_D^{29} = +1.59°$ (from 2-methyl-1-butanol, $[\alpha]_D^{29} = -2.72°$). 75 gm. of paraformaldehyde were added and the mixture stirred overnight. The Grignard reagent was decomposed with ice and hydrochloric acid and the carbinol extracted with ether. B.p. 80° at 47 mm.; yield, 90 gm.; $n_D^{25} = 1.4178$; D $\frac{27}{4} = 0.822$.

$$[\alpha]_D^{27} = \frac{+1.37°}{1 \times 0.822} = +1.67°. \quad [M]_D^{27} = +1.70° \text{ (homogeneous)}$$

2.805 mg. substance: 7.240 mg. CO_2 and 3.400 mg. H_2O.
$C_6H_{14}O$.   Calculated.   C 70.52, H 13.82
        Found.   " 70.40, " 13.56

Levo-2-Bromopentane—65 gm. of methylpropyl carbinol, $[\alpha]_D^{25} = +3.28°$, were cooled in ice and saturated with hydrogen bromide. The solution was heated on a steam bath 1 hour, cooled, and saturated again with hydrogen bromide. The aqueous layer was separated and the 2-bromopentane shaken with cold concentrated sulfuric acid, dilute sodium carbonate solution, then water. It was then fractionated. B.p. 117° at 760 mm.; yield, 85 gm.; D $\frac{25}{4} = 1.208$.

$$[\alpha]_D^{25} = \frac{-7.06°}{1 \times 1.208} = -5.84°. \quad [M]_D^{25} = -8.83° \text{ (homogeneous)}$$

4.290 mg. substance: 6.330 mg. CO_2 and 2.875 mg. H_2O.
$C_5H_{11}Br$.   Calculated.   C 39.73, H 7.34
        Found.   " 40.24, " 7.50

Levo-2-Propylbutyric Acid (4) (β-Methyl Caproic Acid)—8 gm. of sodium were dissolved in 100 cc. of absolute alcohol and 55 gm. of ethyl malonate were added. To this solution 50 gm. of 2-bromopentane, $[\alpha]_D^{25} = -5.84°$, were added. The product was refluxed 3 hours, then poured into water. The malonic ester was extracted

with ether and distilled.   It was hydrolyzed by boiling 2 hours
with 3 mols of potassium hydroxide in 80 per cent alcohol.   The
alcohol was evaporated and the potassium salt acidified with
dilute sulfuric acid.   The malonic acid was extracted with ether
and dried with anhydrous sodium sulfate.   The ether was evapo-
rated and the residue heated in a metal bath at 190° until carbon
dioxide ceased coming off.   It was then distilled under reduced
pressure.   The distillate was dissolved in sodium carbonate solu-
tion and freed from impurities by extraction with ether.   The
carbonate solution was then acidified and the acid extracted with
ether and fractionated.   B.p. 112° at 16 mm.; yield, 18 gm.; D $\frac{26}{4}$
= 0.912.

$$[\alpha]_D^{26} = \frac{-0.46°}{1 \times 0.912} = -0.50°. \quad [M]_D^{26} = -0.66° \text{ (homogeneous)}$$

4.710 mg. substance: 11.225 mg. CO_2 and 4.525 mg. H_2O.
$C_7H_{14}O_2$.   Calculated.   C 64.57, H 10.84
Found.   " 64.99, " 10.75

Levo-3-Bromohexane—100   gm.   of   ethyl-*n*-propyl   carbinol,
$[\alpha]_D^{22} = +1.73°$, were converted into the bromide as described for
2-bromopentane.   B.p. 142° at 760 mm.; D $\frac{22}{4}$ = 1.166.

$$[\alpha]_D^{22} = \frac{-2.37°}{1 \times 1.166} = -2.03°. \quad [M]_D^{22} = -3.35° \text{ (homogeneous)}$$

5.572 mg. substance: 8.978 mg. CO_2 and 3.978 mg. H_2O.
$C_6H_{13}Br$.   Calculated.   C 43.64, H 7.94
Found.   " 43.94, " 7.99

3-Propyl Valeric Acid (5)—60 gm. of 3-bromohexane, $[\alpha]_D^{22} = -2.03°$, were condensed with ethyl malonate as described for
2-propylbutyric acid (4).   The acid obtained was completely
racemized.

Dextro-3-Propyl Valeric Acid (5)—The inactive acid was pre-
pared from 3-bromohexane and ethyl malonate.   228 gm. of the
inactive acid were dissolved in 1 liter of hot acetone and 650 gm. of
quinine were added.   The solution was filtered and allowed to
crystallize in a refrigerator at 0°.   It required 2 days for crystal-
lization.   After five crystallizations the salt was decomposed with

10 per cent hydrochloric acid and the organic acid extracted with ether. B.p. $106°$ at 5 mm.; $D_4^{30} = 0.911$; $n_D^{25} = 1.4287$.

$$[\alpha]_D^{30} \quad \frac{+1.69°}{1 \times 0.911} = +1.86°. \quad [M]_D^{30} = +2.67° \text{ (homogeneous)}$$

4.375 mg. substance: 10.680 mg. CO_2 and 4.510 mg. H_2O.
$C_8H_{16}O_2$. Calculated. C 66.62, H 11.19
Found. " 66.58, " 11.53

Dextro-Ethyl Ester of 3-Propyl Valeric Acid (5)—65 gm. of 3-propyl valeric acid (5), $[\alpha]_D^{30} = +1.86°$, were mixed with 150 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. The mixture was heated 1 hour on a steam bath. Then the excess alcohol was distilled off. The residue was shaken with dilute sodium carbonate solution, extracted with ether, and distilled. B.p. $80°$ at 9 mm.; yield, 67 gm.; $D_4^{30} = 0.866$; $n_D^{25} = 1.4183$.

$$[\alpha]_D^{30} = \frac{+0.51°}{1 \times 0.866} = +0.59°. \quad [M]_D^{30} = +1.02° \text{ (homogeneous)}$$

6.075 mg. substance: 15.405 mg. CO_2 and 6.425 mg. H_2O.
$C_{10}H_{20}O_2$. Calculated. C 69.71, H 11.60
Found. " 69.16, " 11.83

Dextro-3-Ethyl-1-Hexanol—60 gm. of the ethyl ester of 3-propyl valeric acid (5), $[\alpha]_D^{30} = +0.59°$, were added to 300 cc. of absolute alcohol and reduced by dropping into a suspension of sodium in boiling toluene.[1] B.p. $73°$ at 15 mm.; $D_4^{28} = 0.829$; $n_D^{25} = 1.4323$.

$$[\alpha]_D^{28} = \frac{+0.41°}{1 \times 0.829} = +0.49°. \quad [M]_D^{28} = +0.64° \text{ (homogeneous)}$$

This carbinol was purified through its half phthalic ester, but the rotation did not change.

5.225 mg. substance: 14.090 mg. CO_2 and 6.460 mg. H_2O.
$C_8H_{18}O$. Calculated. C 73.78, H 13.94
Found. " 73.55, " 13.83

Dextro-1-Chloro-3-Ethyl Hexane—10 gm. of 3-ethyl-1-hexanol, $[\alpha]_D^{28} = +0.49°$, were cooled in ice and 40 gm. of thionyl chloride were added. The mixture was heated 1 hour on a steam bath,

then fractionated. B.p. 85° at 40 mm.; D $\frac{27}{4}$ = 0.879; n_D^{25} = 1.4335.

$$[\alpha]_D^{27} = \frac{+1.01°}{1 \times 0.879} = +1.15°. \quad [M]_D^{27} = +1.68° \text{ (homogeneous)}$$

0.1559 gm. substance: 0.150 gm. AgCl (Carius).
$C_8H_{15}Cl$. Calculated. Cl 24.19. Found. Cl 23.80

Dextro-1-Bromo-3-Ethyl Hexane—To 10 gm. of 3-ethyl-1-hexanol, $[\alpha]_D^{28}$ = +0.49°, cooled in ice, 20 gm. of phosphorus tribromide were added. The product was heated ½ hour on a steam bath, then poured on ice. The oily layer was shaken with concentrated sulfuric acid, then water, and dried with anhydrous sodium sulfate. B.p. 94° at 35 mm.; yield, 9 gm.; D $\frac{22}{4}$ = 1.119.

$$[\alpha]_D^{22} = \frac{+1.14°}{1 \times 1.119} = +1.02°. \quad [M]_D^{22} = +1.97° \text{ (homogeneous)}$$

Levo-3-Bromoheptane—130 gm. of ethyl-*n*-butyl carbinol, $[\alpha]_D^{22}$ = +7.37°, were converted into the bromide as described for 2-bromopentane. B.p. 79° at 40 mm.; yield, 193 gm.; D $\frac{22}{4}$ = 1.139.

$$[\alpha]_D^{22} = \frac{-10.42°}{1 \times 1.139} = -9.15°. \quad [M]_D^{22} = -16.38° \text{ (homogeneous)}$$

4.505 mg. substance: 7.815 mg. CO_2 and 3.340 mg. H_2O.
$C_7H_{15}Br$. Calculated. C 46.93, H 8.45
Found. " 47.31, " 8.29

Levo-3-Butyl Valeric Acid (5)—To 26 gm. of sodium in 300 cc. of absolute alcohol were added 180 gm. of ethyl malonate and 193 gm. of 3-bromoheptane, $[\alpha]_D^{22}$ = −9.15°. The procedure was the same as described for 2-propylbutyric acid (4).[1] B.p. 130° at 12 mm.; yield, 71 gm.; D $\frac{22}{4}$ = 0.908.

$$[\alpha]_D^{22} = \frac{-1.22°}{1 \times 0.908} = -1.35°. \quad [M]_D^{22} = -2.13° \text{ (homogeneous)}$$

3.165 mg. substance: 7.995 mg. CO_2 and 3.130 mg. H_2O.
$C_9H_{18}O_2$. Calculated. C 68.31, H 11.47
Found. " 68.89, " 11.06

Levo-Ethyl Ester of 3-Butyl Valeric Acid (5)—71 gm. of 3-butyl valeric acid (5), $[\alpha]_D^{22} = -1.34°$, were mixed with 200 cc. of absolute alcohol and 6 cc. of concentrated sulfuric acid. Esterification was carried out as described for the ethyl ester of 3-propyl valeric acid (5). B.p. 107° at 25 mm.; yield, 73 gm.; $D\,_4^{22} = 0.866$.

$$[\alpha]_D^{22} = \frac{-0.49°}{1 \times 0.866} = -0.56°. \quad [M]_D^{22} = -1.05° \text{ (homogeneous)}$$

4.312 mg. substance: 11.210 mg. CO_2 and 4.565 mg. H_2O.

$C_{11}H_{22}O_2$. Calculated. C 70.89, H 11.91

Found. " 70.90, " 11.84

Levo-3-Ethyl-1-Heptanol—60 gm. of ethyl ester of 3-butyl valeric acid (5), $[\alpha]_D^{22} = -0.56°$, were dissolved in 300 cc. of absolute alcohol. This solution was dropped into a suspension of 120 gm. of sodium in 600 cc. of toluene as described for 3-ethyl-1-hexanol. B. p. 101° at 16 mm.; yield, 33 gm. (after purification through its half phthalic ester); $D\,_4^{23} = 0.834$.

$$[\alpha]_D^{23} = \frac{-0.55°}{1 \times 0.834} = -0.66°. \quad [M]_D^{23} = -0.95° \text{ (homogeneous)}$$

3.352 mg. substance: 9.170 mg. CO_2 and 4.175 mg. H_2O.

$C_9H_{20}O$. Calculated. C 74.89, H 13.98

Found. " 74.61, " 13.94

Levo-1-Bromo-3-Ethyl Heptane—To 30 gm. of 3-ethyl-1-heptanol $[\alpha]_D^{23} = -0.66°$, cooled in ice, 60 gm. of phosphorus tribromide were added. The mixture was heated 1 hour on a steam bath, then poured on ice. The oily layer was shaken with concentrated sulfuric acid, then with a sodium carbonate solution, and finally dried and fractionated. B.p. 90° at 15 mm.; yield, 35 gm.; $D\,_4^{23} = 1.103$.

$$[\alpha]_D^{22} = \frac{-0.35°}{1 \times 1.103} = -0.32°. \quad [M]_D^{22} = -0.66° \text{ (homogeneous)}$$

5.015 mg. substance: 9.575 mg. CO_2 and 4.125 mg. H_2O.

$C_9H_{19}Br$. Calculated. C 52.15, H 9.25

Found. " 52.07, " 9.20

Levo-3-Bromooctane—120 gm. of ethyl-n-amyl carbinol, $[\alpha]_D^{22} = +6.82°$, were converted into the bromide as described for 2-bromopentane. B.p. 85° at 25 mm.; yield, 166 gm.; $D\,_4^{22} = 1.097$.

$$[\alpha]_D^{22} = \frac{-12.11°}{1 \times 1.097} = -11.04°. \quad [M]_D^{22} = -21.32° \text{ (homogeneous)}$$

5.934 mg. substance: 10.960 mg. CO_2 and 4.735 mg. H_2O.
$C_8H_{17}Br$. Calculated. C 49.72, H 8.88
Found.   " 50.37, " 8.93

Levo-3-n-Amyl Valeric Acid (*5*)—23 gm. of sodium were dissolved in 200 cc. of absolute alcohol and 160 gm. of ethyl malonate and then 160 gm. of 3-bromooctane, $[\alpha]_D^{22} = -11.04°$, were added to the solution. The acid was prepared and purified as described for 2-propylbutyric acid (4). B.p. 140° at 12 mm.; yield, 55 gm.; $D_4^{22} = 0.899$.

$$[\alpha]_D^{22} \quad \frac{-1.50°}{1 \times 0.899} = -1.67°. \quad [M]_D^{22} = -2.87° \text{ (homogeneous)}$$

2.759 mg. substance: 7.070 mg. CO_2 and 2.975 mg. H_2O.
$C_{10}H_{20}O_2$. Calculated. C 69.69, H 11.71
Found.   " 69.89, " 12.06

Levo-Ethyl Ester of 3-n-Amyl Valeric Acid (*5*)—55 gm. of 3-n-amyl valeric acid (5) were dissolved in 150 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid added. Esterification was carried out as described for the ethyl ester of 3-propyl valeric acid (5). B.p. 104° at 12 mm.; yield, 59 gm.; $D_4^{22} = 0.865$.

$$[\alpha]_D^{22} = \frac{-0.66°}{1 \times 0.865} = -0 76°. \quad [M]_D^{22} = -1.53° \text{ (homogeneous)}$$

4.705 mg. substance: 12.460 mg. CO_2 and 5.010 mg. H_2O.
$C_{12}H_{24}O_2$. Calculated. C 71.93, H 12.08
Found.   " 72.22, " 11.91

Levo-3-Ethyl-1-Octanol—59 gm. of ethyl ester of 3-n-amyl valeric acid (5), $[\alpha]_D^{22} = -0.76°$, were dissolved in 250 cc. of absolute alcohol and this dropped into a suspension of 100 gm. of sodium in 500 cc. of boiling toluene. The reduction was carried out as described for 3-ethyl-1-hexanol. B.p. 110° at 15 mm.; yield, 30 gm.; $D_4^{22} = 0.833$.

$$[\alpha]_D^{22} = \frac{-0.96°}{1 \times 0.833} = -1.15°. \quad [M]_D^{22} = -1.82° \text{ (homogeneous)}$$

3.689 mg. substance: 10.220 mg. CO_2 and 4.675 mg. H_2O.
$C_{10}H_{22}O$.   Calculated.   C 75.85, H 14.02
Found.   " 75.56, " 14.18

Levo-1-Bromo-3-Ethyl Octane—25 gm. of 3-ethyl-1-octanol, $[\alpha]_D^{22} = -0.61°$, were cooled in ice and 50 gm. of phosphorus tribromide were added. Bromination was completed as described for 1-bromo-3-ethyl heptane. B.p. 99° at 14 mm.; yield, 29 gm.; D $_4^{22}$ = 1.079.

$$[\alpha]_D^{22} \quad \frac{-0.66°}{1 \times 1.079} \quad -0.61°. \quad [M]_D^{22} = -1.35° \text{ (homogeneous)}$$

3.282 mg. substance: 6.550 mg. CO_2 and 2.670 mg. H_2O.
$C_{10}H_{21}Br$.   Calculated.   C 54.27, H 9.57
Found.   " 54.43, " 9.10

Dextro-6-Ethyl-3-Nonanol—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 37 gm. of 1-chloro-3-ethyl hexane, $[\alpha]_D^{27} = +1.15°$. This was cooled in ice and 20 gm. of propionaldehyde slowly added. The Grignard reagent was decomposed and the carbinol extracted in the usual manner. B.p. 114° at 16 mm.; yield, 31 gm.; D $_4^{22}$ = 0.830.

$$[\alpha]_D^{22} = \frac{+0.27°}{1 \times 0.830} = +0.32°. \quad [M]_D^{22} = +0.56° \text{ (homogeneous)}$$

3.876 mg. substance: 10.863 mg. CO_2 and 4.936 mg. H_2O.
$C_{11}H_{24}O$.   Calculated.   C 76.65, H 14.05
Found.   " 76.44, " 14.25

Dextro-4-Ethyl Nonane—30 gm. of 6-ethyl-3-nonanol, $[\alpha]_D^{22} = +0.32°$, were heated under reflux with 200 gm. of "constant boiling" hydriodic acid for 15 minutes. The iodide was extracted with ether and the ether evaporated under reduced pressure. The residue was mixed with 100 gm. of finely divided zinc and reduced by adding concentrated hydrochloric acid. The hydrocarbon was extracted and purified as described previously.[1] B.p. 77° at 20 mm.; yield, 8 gm.; D $_4^{23}$ = 0.745.

$$[\alpha]_D^{22} = \frac{+0.52°}{1 \times 0.745} = +0.70°. \quad [M]_D^{22} = +1.09° \text{ (homogeneous)}$$

3.649 mg. substance: 11.242 mg. CO_2 and 5.029 mg. H_2O.
$C_{11}H_{24}$.   Calculated.   C 84.51, H 15.49
          Found.   " 84.02, " 15.42

Levo-4-Ethyl-1-Nonanol—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 75 gm. of 3-ethyl-1-bromooctane, $[\alpha]_D^{22} = -0.69°$. To the Grignard reagent were added 15 gm. of paraformaldehyde and the mixture was stirred 20 hours. The Grignard reagent was decomposed and the carbinol worked up as usual. B.p. 127° at 15 mm.; yield, 20 gm.

$$[\alpha]_D^{23} = 0° \text{ (homogeneous)}$$

4.120 mg. substance: 11.644 mg. CO_2 and 5.215 mg. H_2O.
$C_{11}H_{24}O$.   Calculated.   C 76.66, H 14.05
           Found.   " 77.08, " 14.16

Levo-4-Ethyl Nonane—20 gm. of 4-ethyl-1-nonanol, $[\alpha]_D^{23} = 0°$, were brominated by 25 gm. of phosphorus tribromide. The halide was isolated as previously described. B.p. 122° at 15 mm.; yield, 24 gm.; $D \frac{22}{4} = 1.054$.

$$[\alpha]_D^{22} = \frac{+\ 0.62°}{2 \times 1.054} = +\ 0.29°. \quad [M]_D^{22} = +\ 0.71° \text{ (homogeneous)}$$

24 gm. of halide were reduced by forming a Grignard reagent with 3 gm. of magnesium in dry ether. This was poured onto ice and the hydrocarbon purified as previously described. B.p. 77° at 20 mm.; yield, 7 gm.; $D \frac{23}{4} = 0.745$.

$$[\alpha]_D^{23} = \frac{-\ 0.74°}{2 \times 0.745} = -\ 0.50°. \quad [M]_D^{23} = -\ 0.76° \text{ (homogeneous)}$$

2.834 mg. substance: 8.738 mg. CO_2 and 3.920 mg. H_2O.
$C_{11}H_{24}$.   Calculated.   C 84.51, H 15.49
          Found.   " 84.09, " 15.48

NUTRITIVE VALUE OF POTATO PROTEIN AND OF GELATIN

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Notwithstanding the extensive use of the potato as a food in many countries and throughout many generations, there exists a difference of opinion among investigators regarding the nutritive value of the potato protein.

Metabolism experiments conducted with human subjects have generally yielded results which indicate that the potato proteins have a high nutritive value. Hinhede (1) found that a man can retain full vigor for a year or longer on a diet of potatoes and fat. Thomas (2), using steamed or baked potatoes, found that the proteins had an average biological value of 78.9 per cent. Rose and Cooper (3) showed that for human subjects the nitrogen of the potato is of extraordinary value for replacing nitrogen lost through daily catabolism in the adult. More recently, Kon and Klein (4) described an experiment in which two adults lived over a period of 167 days in nitrogen equilibrium and in good health on a diet in which practically all the nitrogen was derived from the potato.

Feeding experiments in which potatoes were fed to rats have yielded less favorable results. McCollum, Simmonds, and Parsons (5), using a potato diet furnishing from 7 to 8 per cent of protein, found that potato nitrogen is of no greater value for growth than an equivalent amount of protein from one of the cereal grains.

Hartwell (6) found that when rats were fed a diet containing cooked potato as the sole source of nitrogen the animals grew at a very unsatisfactory rate. The poor results obtained were attributed to the low level of protein in the diet (not more than 7.6 per cent) rather than to the quality of the protein.

Kon (7) recently reported the results of feeding experiments with rats in which tuberin, the isolated protein of the potato, was fed. From the satisfactory rate at which the animals grew when the diet contained 8 to 9 per cent of tuberin, he concluded that the protein was of good nutritive quality.   It should be noted, however, that he used an aqueous extract of yeast to supply vitamin B. This introduced extraneous nitrogen which might well have affected the results of the feeding experiments.

It is very probable that some of the poor results reported which have been obtained when potatoes were fed as the sole source of dietary nitrogen are to be attributed to the low level of protein. On account of the high percentage of starch, and the presence of other non-nitrogenous constituents of potatoes, it is impossible, when the whole potato is used, to prepare a potato diet having more than 7 to 8 per cent of crude protein.   This low level of nitrogen becomes even more significant when it is borne in mind that only about 6⅓ per cent of potato nitrogen is reported to be in the form of protein.

In the experiments described in this paper, a potato preparation was used which contained but a small amount of starch.   It was believed that the use of such a preparation in feeding experiments to determine the nutritive value of potato protein would have a twofold advantage: by making it possible to feed the potato protein at a higher level of intake, and to eliminate the possibility of any unfavorable effects resulting from the high percentage of starch in the diet.

The fact that no growth resulted when the potato preparation was fed to rats as the sole source of dietary nitrogen, even when supplemented both with gelatin up to 20 per cent and with tryptophane, tyrosine, and cystine, amino acids which are either absent in gelatin or present only in traces, indicates that both the potato preparation and gelatin are lacking or deficient in some dietary factor that is present in casein and lactalbumin.

The following figures show that tuberin contains sufficient cystine and histidine, amino acids which are lacking or deficient in gelatin and which are known to be essential for the satisfactory growth of animals.   The percentage of tryptophane in tuberin is not known.

Amino acids	Gelatin	Tuberin
	per cent	*per cent*
Lysine...............................	5.9	3.3
Tyrosine..............................	Trace	4.3
Cystine...............................	"	4.4
Tryptophane..........................	0.0	
Histidine.............................	0.9	2.3
Arginine.............................	8.0	4.2

In their study on the nutritive properties of gelatin, Jackson, Sommer, and Rose (8) have shown that in addition to supplying tyrosine, tryptophane, cystine, and histidine, supplementing the gelatin diet with glutamic acid, aspartic acid, valine, phenylalanine, leucine, isoleucine, and alanine produced no better results than gelatin alone. Diets containing hydrolyzed gelatin supplemented with amino acids did not support growth better than similar diets containing unhydrolyzed gelatin.

Osborne and Mendel (9) found that rats declined rapidly when fed a diet containing gelatin as the sole source of protein, and that they recovered when one-half of the gelatin was replaced by wheat gliadin, a protein incapable of inducing more than a very slight growth when it constitutes the sole protein constituent of the diet. They also found that when the gelatin in the diet was either partially or entirely replaced by casein, decline in weight was arrested and followed by maintenance and repair.

The poor results obtained in our feeding experiments on rats with the potato preparation are not in agreement with those of investigators who have conducted metabolism experiments with human subjects, and who found the potato proteins to have satisfactory nutritive properties. In explanation of this discrepancy it may be postulated that some hitherto unidentified amino acid of a labile character, which is present in potatoes but lacking in gelatin, was destroyed during the process of preparation of the potato material. It may be that an enzyme acted on some unknown but nutritionally essential factor in a manner similar to the well known action of tyrosinase on tyrosine in potato juice.

Evaluation of the nutritive properties of potato protein would be more conclusive if the potato preparation itself contained enough vitamins B and G so that addition of yeast to the diet to

supply these vitamins could be omitted, thus avoiding the intro-
duction of extraneous nitrogen. We found that with 20 per cent
of the potato preparation as the sole source of vitamins B and G
in the diet rats grew as rapidly as when they received 5 per cent or
more dried yeast.

Materials Used

Biological tests were made on young albino rats taken from our
stock colony. The animals were kept in individual cages provided
with screen bottoms to prevent access to excreta. They were
weighed twice each week and records of their food consumption
were maintained. The basal diet had the following composition:

Potato preparation.	41.3	parts
Salt mixture.	3.0	"
Dextrin.	53.7	"
Cod liver oil.	2.0	"

This ration provided 9.0 per cent of crude potato protein. The
salt mixture had the following composition:

Dicalcium phosphate.	430	parts
Sodium chloride.	88	"
Potassium chloride.	50	"
" sulfate.	55	"
Ferric citrate.	5	"
Potassium iodide.	1	part

Potato Preparation—Irish Cobbler potatoes, in 14 kilo lots, were
washed and finely pulped in an electrically driven grinding mill.
The pulp, in several separate portions, was placed in a finely-
meshed screen basket, and most of the starch was washed out by
repeatedly dipping the basket in a bucket of distilled water.
Three fresh portions of water were used. After the starch had
settled the clear liquid was decanted, and the starch washed twice
or three times with fresh water. One-half of the pulp was squeezed
by hand in a muslin bag in order to remove as much liquid as pos-
sible. · The residue was then washed with distilled water and the
liquid again expressed as before. The liquid and washings were
added to the main potato liquid, but the washed pulp was dis-

carded.[1] The other half of the pulp was returned unwashed to the joint liquids and washings. Addition of this proportion of the pulp was found to facilitate drying of the product, and gave to it a desirable bulk. The mixture was then concentrated at 96-97° to a small volume in a steam-jacketed kettle. The concentrate was finally dried at 50-65° in trays in a drier, and the residue was ground to a fine powder. The ashen gray product had but little starch, and it contained 3.48 per cent nitrogen.

Dextrin was prepared by autoclaving moistened corn-starch for 2 hours at 15 pounds pressure. The product was then dried and ground. Cod liver oil of known high vitamin potency was added to such amounts of the diet as would be consumed in 1 week. When other materials were included in the diet, they replaced an equal weight of dextrin.

In order to determine the nature of the growth-limiting factor in the potato preparation, the following materials were fed:

Casein—A commercial product that had been thoroughly extracted with hot alcohol for vitamin studies (10).

Yeast—Dry yeast[2] compressed in the laboratory to tablets of the desired weight for feeding. 5 per cent of this yeast as the sole source of vitamins B and G permitted growth at the maximum rate in our animals.

Autoclaved Yeast—The yeast mentioned above, which had been autoclaved for 4 hours at 15 pounds pressure, and found to be devoid of vitamin B but to contain substantial amounts of vitamin G.

Gelatin—Gold Seal brand.

Tyrosine, Tryptophane, and Cystine—Preparations of high purity used as standards for colorimetric determinations of these amino acids in this laboratory.

EXPERIMENTAL RESULTS AND DISCUSSION

When fed the basal diet alone the rats nearly maintained their weight for a period of 3 weeks. 10 per cent of casein added to the diet produced an immediate growth response which stopped, however, when the casein was withdrawn from the diet 4 weeks later.

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[1] The discarded starch and pulp when dried, contained respectively 0.06 and 0.55 per cent nitrogen.

[2] The yeast used in the work was obtained from the Northwestern Yeast Company.

4 per cent yeast, untreated, or autoclaved to destroy vitamin B, did not produce any change in the growth rate when added to the basal diet. The deficiency in our basal diet was, therefore, in its protein, and we then attempted to demonstrate whether it was quality or quantity of protein or both. When 10 per cent of gelatin proved entirely incapable of making up the deficiency, we suspected that the addition of tyrosine, tryptophane, or cystine, or a combination of these amino acids, would make the diet adequate for growth. However, they proved no better than gelatin.

TABLE I

Growth Response to Addition of Various Substances to the Basal Potato Diet

Experiment No.	Addition* to basal potato diet	Growth response
	per cent	
1	None	Maintenance
2	10 casein, 4 yeast	Normal growth
3	10 "	" "
4	10 lactalbumin	" · "
5	4 yeast	Maintenance
6	4 autoclaved yeast	"
7	10 gelatin	"
8	0.5 tryptophane, 2.0 tyrosine	"
9	0.5 " 2.0 " 0.2 cystine	"
10	10 gelatin, 0.5 tryptophane, 2.0 tyrosine, 0.2 cystine	"
11	20 gelatin, 0.5 tryptophane, 2.0 tyrosine, 0.2 cystine	"

* Additions to the basal diet were made at the expense of an equal amount of dextrin.

In order to ascertain whether the diet was satisfactory, we included in it 10 per cent of gelatin, 2.0 per cent of tyrosine, 0.5 per cent of tryptophane, and 0.2 per cent of cystine. This combination proved inadequate for growth, and increasing the gelatin to 20 per cent did not improve it. The results are summarized in Table I, and data are given showing that both purified casein and lactalbumin supplied to the basal potato diet some·component necessary for growth which is not one of the known essential amino acids. When fed the basal potato diet the animals experienced a uniform loss in weight of a few gm. in 20 days (see Chart I). In the other

cases where we report maintenance there was a uniform increase in weight of slightly greater magnitude.   While these slight differences in response may be significant, they are in marked contrast to the rapid growth produced by adding casein to the diet.   Normal growth is used to designate maximum rate of growth of the animals.
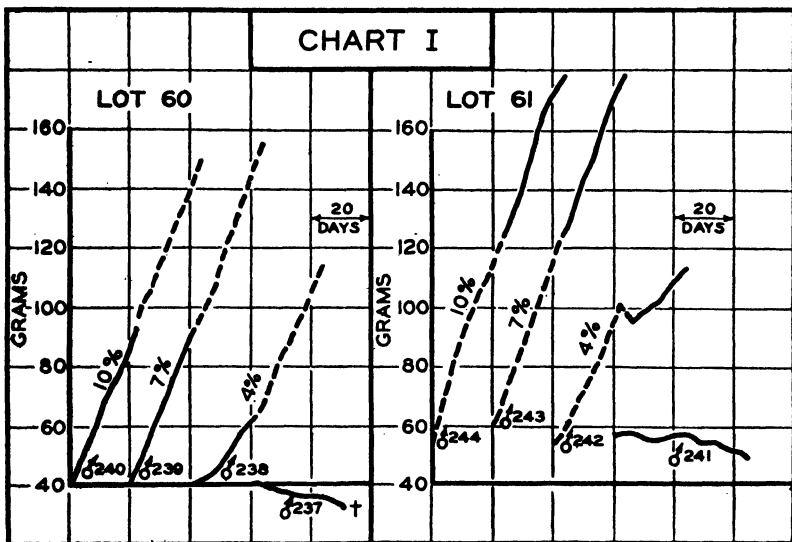
CHART I. These curves show that lactalbumin is more efficient than casein in making up the deficiency in our potato preparation when 4 per cent of these proteins is added to the basal potato diet described in the text. At the levels of 7 and 10 per cent these proteins are equally efficient.   The broken curves indicate the periods over which the lactalbumin was fed. The figure to the left of each curve indicates the level of casein or lactalbumin fed.   Rats 237 and 241 received the basal potato diet only and their growth curves are typical of the performance of our rats on this diet.

Having established that the deficiency in our basal diet was not one of quantity of protein and that casein provided some dietary essential not found in gelatin, nor one of the known essential amino acids in which gelatin is known to be deficient, we thought that some data on the relative quantities in casein and lactalbumin of the missing entity might help reveal its identity.   Accordingly,

these proteins were fed at levels of 4, 7, and 10 per cent in the basal diet. The results are recorded in Chart I. In one case (Lot 60) four male rats, litter mates, were fed the following diets: Rat 237, basal diet; Rat 238, basal diet containing 4 per cent casein; Rat 239, basal diet containing 7 per cent casein; Rat 240, basal diet containing 10 per cent casein. At the end of 22 days the casein was replaced by an equal quantity of lactalbumin. In another case (Lot 61) this experiment was duplicated except that the order of feeding casein and lactalbumin was reversed. The performance of the animals showed that there was no significant difference between the two proteins when fed on the 7 and 10 per cent levels, but 4 per cent of lactalbumin as an adjunct was definitely superior to the same level of casein.

SUMMARY

Feeding experiments were conducted with rats with the object of ascertaining the nutritive value of potato protein. The material fed was a preparation containing 3.48 per cent nitrogen and representing all the constituents of the potato except most of the starch and one-half of the washed pulp. When this preparation, fed at a level of 9 per cent of crude protein, furnished the sole source of nitrogen in a diet adequate with respect to the essential dietary factors other than protein, only maintenance resulted. Addition to the diet of as much as 20 per cent gelatin, either alone or together with a mixture of cystine (0.2 per cent), tyrosine (2.0 per cent), and tryptophane (0.5 per cent) caused no improvement in the rate of growth. On the other hand, addition of 10 per cent casein or lactalbumin enabled the animals to grow at a normal rate.

It is concluded that casein and lactalbumin contain some essential dietary factor which was lacking in the potato preparation and in gelatin, and which is not one of the known essential amino acids.

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STUDIES OF THE METABOLISM OF WOMEN

V. THE COMPONENTS CONCERNED IN THE CYCLIC VARIATIONS IN THE LEVEL OF TOTAL NON-PROTEIN NITROGEN IN THE BLOOD OF NORMAL WOMEN*

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Several years ago the writers published evidence that high values for non-protein nitrogen of circulating blood in normal women were frequent at or near the time of onset of menstruation. Likewise we published at that time data which indicated that, while blood uric acid values were likely to be high preceding and following the time of onset of menstruation, they were frequently low at the beginning of the menstrual period. At that time the number of our observations of concentration of creatine and creatinine, urea, amino acids, etc., did not justify any conclusions as to the fraction of the non-protein nitrogen involved in the menstrual increase in total non-protein nitrogen. Moreover we did not then fully realize the necessity for making observations on blood composition at equal intervals throughout the whole month for statistical comparison, nor had we been able to secure an adequate series of control subjects.

The search for the non-protein nitrogenous constituent of blood responsible for this large menstrual variation in total non-protein nitrogen has been continued when the time and opportunity have been available and the data now accumulated seem to justify a further brief report, with certain tentative conclusions.

Methods

The general plan of observation has already been described (1). The subjects were healthy university students, men and women, and a few healthy women past the age of menopause.   Blood samples for all of the later series were taken before breakfast at least three times a week.   Folin-Wu protein-free filtrates (2) made from laked blood were used.   In most of the later series of analyses, the Koch-McMeekin technique (3) for non-protein nitrogen was substituted for the Folin-Wu procedure.   Uric acid was determined
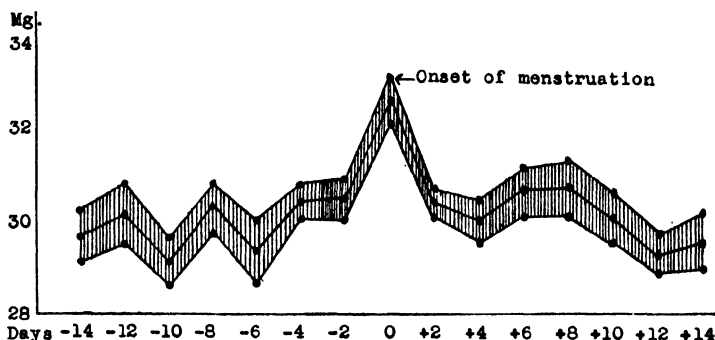
Fig. 1. Composite curve for non-protein nitrogen made by dividing the month into fourteen 48 hour intervals.   The mean of all observations for each interval is indicated by a point in the center line, while the probable errors of these means are indicated by the shaded areas.   Total, 600 determinations, forty-two subjects.

by the direct procedure of Benedict (4).   Amino acids were determined both by the methods of Van Slyke (5) and of Folin (6). The adenine nucleotide figures used in the statistical comparison were determined by the method of Jackson (7), since the method of Buell proved unsatisfactory with the amounts of blood filtrate which we could dedicate to this analysis.

Data—Presentation of the original figures for the blood analyses would have required far too much space.[1]   We have therefore divided the monthly cycle into fourteen 48 hour periods and deter-

[1] Original figures are on file in the dissertation of Statie E. Erikson in the University of California Library.

mined the mean, and, where the day to day variation justified it, the probable error of the mean of all the data for each blood constituent determined during each period.   48 hours were taken as
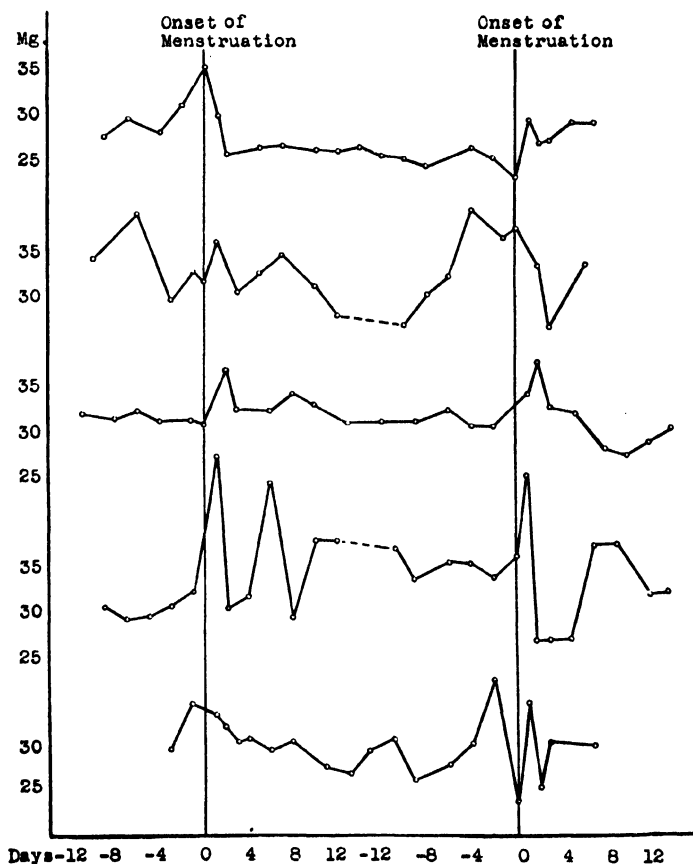
FIG. 2. Representative individual curves for total non-protein nitrogen showing variation in time of greatest increase in values for total non-protein nitrogen in relation to onset of menstruation.

the average interval between observations.   We believe this is as often as blood samples of the size required can be taken without risk of interference with the health of the subjects.

In Fig. 1 the points in the center line represent the means of all

our non-protein nitrogen figures for each interval while the shaded
areas show the probable errors of those means computed by the
usual method.   It will be seen that the rise in total non-protein
nitrogen at the beginning of the menstrual period is statistically
highly significant, while a secondary rise followed by a low point
about 9 days before this time suggests a possible effect of ovulation
on blood non-protein nitrogen.   This latter low point in the curve
is hardly to be rated as statistically significant when considered
alone.   But as will be seen by inspection of Fig. 2 any composite
curve of menstrual variations such as that in Fig. 1, will fail to
give an entirely representative picture.   This is because there is
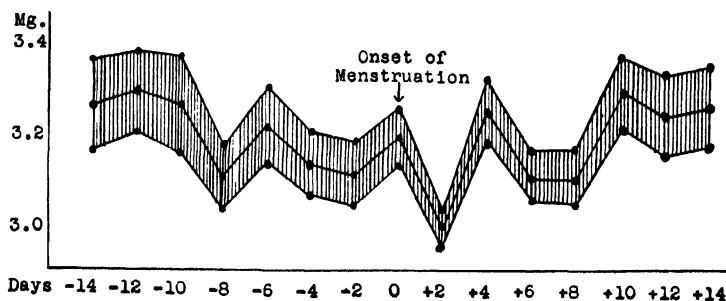
FIG. 3. Composite curve for uric acid made by dividing the month into
fourteen 48 hour intervals.   The mean of all observations for each interval
is indicated by a point in the center line, while the probable errors of the
means are indicated by the shaded areas.   Total, 563 determinations,
forty subjects.

considerable individual difference in the time of appearance of the
high non-protein nitrogen figures in relation to the onset of men-
strual bleeding.   This is entirely in accord with the findings of
Swezy (personal communication) with regard to variations in the
time of onset of estrus in relation to the oogenetic cycle, both in
different individuals of the same species and in the same individuals
at different times.   Individual variation affects to an even larger
. extent the mathematical significance of the composite curve for
uric acid (Fig. 3) which has been plotted by the method described
for Fig. 1.   For individual figures for uric acid the reader is
referred to the previously published paper (1).

    In this connection we should perhaps take into consideration

recent anatomical evidence (8) which indicates that menstrual bleeding sometimes takes place independently of disintegration of endometrial tissue. This would indicate that the mechanisms
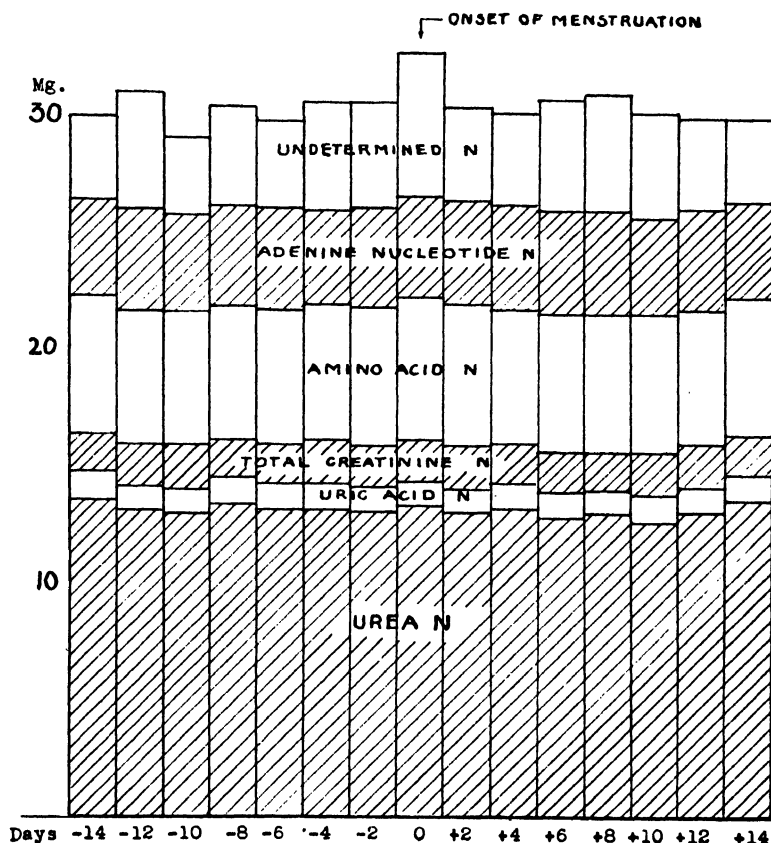
FIG. 4. Distribution of non-protein nitrogenous constituents of blood of normal women. Each parallelogram represents the means of all determinations of each constituent for the 48 hour period indicated. The total numbers of determinations used in computing this figure are as follows:

Total non-protein N .... 600 determinations, 42 subjects
Urea N........................ 423   "   25 series
Uric acid N................... 563   "   40 subjects
Total creatinine N............ 213   "   16   "
Amino acid N.................. 248   "   16   "
Adenine nucleotide N.......... 190   "   16   "

involved in producing the two phenomena are not the same.   But it would also give logical explanation for the fact that the changes in the blood picture differ in different individuals both as to quantity and time interval from the onset of menstruation.   In general we believe that the bulk of our own evidence indicates that the two phenomena are to be regarded as usually nearly simultaneous in the average normal young woman.   .

Menstrual variations in the mean values for the other non-protein nitrogenous constituents of blood are not significant when judged statistically.   Indeed it would seem from even casual inspection that the before breakfast values for urea, creatine and creatinine, amino acids and adenine nucleotides are relatively constant for most individuals.   We have felt, however, that the number of figures which we have has justified us in using the means for each 48 hour period rather than the grand averages in making up the summary of distribution of non-protein nitrogenous constituents in circulating blood at the different stages of the monthly cycle (Fig. 4).

We believe that this composite figure shows even more strikingly than inspection of individual figures, however, that the menstrual variation in concentration of total non-protein nitrogen involves chiefly the "undetermined" fraction.   In other words, this represents a variation in the concentration of some nitrogenous substance or substances of unknown nature.

There is relatively little indication of the nature and function of the unknown fraction of the non-protein nitrogen involved in the menstrual change.   The work of Klaus (9) has suggested that it may be choline formed from the disintegration of lecithin in the tissues involved in the reproductive function.   His findings indicate that choline is excreted in comparatively high concentration in menstrual blood and in perspiration at the time of menstruation. We have shown qualitatively, by the periodide test, that an appreciable amount of choline is present in filtrates from blood taken at the time of the menstrual period, but it is impossible to withdraw the amounts of blood required for quantitative determination of choline by the very unsatisfactory methods at present available.   While the findings with regard to corpus luteum and endometrium in the pig (10) indicate possible menstrual disintegration of lecithin, there is a very large question as to whether the

weight of the tissues involved is sufficient to account for all of the nitrogen as choline. Moreover, our data with regard to phosphate in circulating blood (11) give no indication of the fragmentation of any organic phosphorus compound.

Very early in the course of the present investigation our attention was turned to the possibility that we were dealing with variations in the thioneine (thiasine) content of the blood. But we found, as was stated in an earlier paper (1), no correlation between the time of the menstrual cycle and the times at which we encountered the greatest differences between uric acid figures as determined by the direct method of Benedict and Franke and those determined by the indirect methods of Folin and Wu and of Morris and Macleod (12). We reasoned, therefore, that since thioneine was probably the substance which accounted for the greatest part of the difference in the findings by the two types of methods, variations in the thioneine content of blood were not correlated with the menstrual cycle. Since the experimental work here reported was done, a more reliable method for determination of thioneine has been published by Behre and Benedict (13). Moreover, we have not ruled out the possibility that there may be a menstrual variation in glutathione. Hence further investigation of the fraction of the non-protein nitrogen which reduces the uric acid reagents may be indicated.

There is, however, evidence that glutathione as well as thioneine occurs chiefly in the corpuscles. Unpublished data from this laboratory (Okey and Steinmetz) indicate little or no variation in corpuscle volume (hematocrit index) or in hemoglobin associated with the menstrual cycle. While we wish to repeat this work with a larger number of subjects, we believe that we are justified in assuming, on the basis of data at present available, that if the variation in total non-protein nitrogen of laked blood filtrates which we have observed is a variation in glutathione or thioneine in corpuscles, it must represent a rather improbable variation in concentration within the corpuscles, rather than an increased percentage of corpuscles. Further discussion is postponed until more data are available.

Of the control subjects studied, the men showed about the variation in total non-protein nitrogen and in distribution of non-protein nitrogenous constituents to be expected from the diets

given.   There was no periodicity of high figures.   This was like-
wise true of the two women past menopause whom we were able
to observe.   One young woman suffering from temporary amenor-
rhea whom we happened to observe, showed some periodic varia-
tion in non-protein nitrogen.

SUMMARY AND CONCLUSIONS

1. There is at the time of onset of menstruation in normal women
a rise in total non-protein nitrogen of circulating blood, varying
as a rule from 5 to 15 mg. in individual cases.

2. This increase in concentration of non-protein nitrogen is not
accounted for by urea, uric acid, creatine, creatinine, amino acid,
or adenine nucleotide nitrogen.   Hence it involves chiefly the
"rest" nitrogen.

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COMPOSITION OF BONE

XII. EFFECT OF INADEQUATE AMOUNTS OF VIOSTEROL ON THE HEALING OF RICKETS

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In curative experiments Hess and his collaborators found (1) that "when inadequate amounts of irradiated ergosterol were given to rachitic rats, the phosphate concentration of the blood was raised to the normal level in spite of the fact that no antirachitic or calcifying action was demonstrable in the epiphyses of the long bones."

This curious finding—a rise in phosphorus, following administration of viosterol, unaccompanied by renewal of calcification—was studied in the experiments reported in the present paper.

Experiment I. Small Amounts of Viosterol

Severe rickets was produced in a set of rats by means of Ration 2965 of Steenbock and Black (2). The initial control group gave a value for P of 2.6 and a Ca × P product of 33 (see Table I).

Then viosterol was added to the Steenbock ration in the proportion of 0.001 cc. of viosterol 100 D per 100 gm. of diet. To a second batch of food 0.0005 cc. of viosterol 100 D was added to each 100 gm. of diet. (The viosterol 100 D was first diluted 100 times with olive oil; suitable aliquots were then taken for incorporation in the diet. The values are given in terms of cc. of viosterol 100 D for the sake of comparison with the values of Hess *et al.* (1).) These two test diets were then fed to the ricketic rats for 10 days.

A fourth group of ricketic rats, continued on the Steenbock diet, served as a final control.

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The test period employed by Hess *et al.* was 10 days.   It is seen from Table I that after 10 days the Ca × P products rose to 38 and 43 in the rats which had received viosterol; however, healing of the rickets had also occurred.   Thus a rise in the product unaccompanied by healing was not obtained.   The final control group had a product of 26; no healing had occurred.

Other rats were continued on the viosterol diets for an additional 6 days; the Ca × P products rose somewhat higher and more new calcification was noted.

TABLE I

Effect of Small Amounts of Viosterol on Healing of Rickets

Rat No.	Viosterol 100 D in diet	Experi- mental period	Ca	P	Ca × P	Healing
	cc. per 100 gm.	days	mg. per 100 cc.	mg. per 100 cc.		
376–379	Control	0	12.5	2.6*	33*	—
380–383	0.001	10	12.7	3.4	43	+++
384–387	0.0005	10	12.6	3.0	38	++
388–390	Control	10	10.7	2.4	26	—
391–395	0.001	16	12.6	3.6	45	+++
396–400	0.0005	16	11.6	3.9	45	+++

* Slight hemolysis.

Experiment II.   Inadequate Amounts of Viosterol

The amounts of viosterol employed in Experiment I thus resulted in the usual picture: an increased P and Ca × P product, and the presence of new calcification.   In Experiment II, smaller amounts of viosterol were incorporated in the diet in an attempt to obtain a rise in the Ca × P product without the concomitant occurrence of healing.

Accordingly, three batches of diet were made up as in Experiment I, except that smaller quantities of viosterol were added to the Steenbock diet.   The details are summarized in Table II.

The initial control group had a value for P of 2.2, a Ca × P product of 24, and showed severe rickets.   The various curative diets were then fed for an experimental period of 10 days; one

group was continued on the Steenbock diet and served as a final control.

The final control group had a value for P of 1.5, a Ca $\times$ P product of 15, and showed severe rickets. The group which had received 0.00001 cc. of viosterol[1] per 100 gm. of diet had a Ca $\times$ P product of 22; the group which had received 0.0001 cc. had a Ca $\times$ P product of 28; the group which had received 0.0002 cc. had a Ca $\times$ P product of 29. All of the rats showed severe rickets; no healing was evident in any of them.

TABLE II

Effect of Inadequate Amounts of Viosterol on Healing of Rickets

Rat No.	Viosterol 100 D in diet	Experi-mental period	Ca	P	Ca $\times$ P	Healing
	cc. per 100 gm.	days	mg. per 100 cc.	mg. per 100 cc.		
401–404	Control	0	10.8	2.2	24	—
414–418	0.0002	10	11.3	2.6	29	--
419–423	0.0001	10	11.2	2.5	28	—
424–428	0.00001	10	10.6	2.1	22	—
410–413	Control	10	10.2	1.5	15	—
444, 446	0.0002	13		3.1		—
440, 441 432, 443	0.0001	13	10.6	2.9	31	—
436–439	0.00001	13	11.3	2.6	29	—
433–435	Control	13		2.9		—

The remaining rats were continued on the various diets for an additional 3 days. At this time, a number of rats showed severe paralysis and could not reach the food. The remaining rats were therefore all killed and the paralyzed rats were discarded. The data in Table II show that normal or high products, without healing, were not obtained either after 10 days or after 13 days.

[1] The viosterol 1 D used in Experiment I was diluted 100 times with olive oil; suitable aliquots of this viosterol 0.01 D were then incorporated in the diet. The values in the tables are all given in terms of viosterol 100 D.

Experiment III.   Effect of Dry Milk

In Table I of Hess *et al.* (1) the results of incorporating dry milk in the rickets-producing ration are given.   They reported that daily administration of 10 cc. of reconstituted dry milk produced rickets with no healing; the serum P values were characteristically low.   When very small amounts of viosterol were given in addition to the non-irradiated dry milk, "there was absolute lack of healing . . . and nevertheless the inorganic phosphorus of the blood was found to have risen to a concentration of 6.05."

They stated, "It should be emphasized that this peculiar result, this non-conformity between the classical chemical alteration of the blood and the healing process, was due largely to the fact that the rickets-producing ration contained a considerable percentage of milk, in other words of Ca and P in a favorable ratio.   Had any of the standard rickets-producing rations been employed, minimal amounts of irradiated ergosterol would not have raised the phosphorus in this way."

In Experiment III, therefore, we incorporated dry milk[2] in the Steenbock ration, and included additions of various amounts of viosterol.   The results are summarized in Table III.

The initial control group had a value for P of 2.8, a Ca $\times$ P product of 28, and showed severe rickets.   In addition to the various diets containing dry milk, two groups of rats were included in this experiment in order to have a further check on the potency of the samples of viosterol 100 D and of cod liver oil employed in these studies.   It is seen from Table III that 0.25 cc. of cod liver oil per 100 gm. of the Steenbock diet gave good healing, and that 0.005 cc. of viosterol 100 D also gave good healing in 10 days; the P and Ca $\times$ P values were well above the ricketic level in both groups.

All three groups which had received dry milk in an amount equivalent to 10 cc. of milk daily showed Ca $\times$ P products ranging from 48 to 51; moreover, they all showed considerable healing. The final control group showed a low P value and complete absence of any healing.

[2] The experiments of Hess and Supplee (1, 3) were published from the Research Laboratories of The Dry Milk Company.   We therefore used in these experiments Cremora dry whole milk of The Dry Milk Company. The equivalent of 10 cc. of reconstituted milk was calculated from the stated fat content.

The healing obtained with the diet to which only dry milk had been added was not typical. Instead of having a line of calcification at the provisional zone, the healing took place within the metaphysis. The new calcification was irregular and sporadic; the uncalcified cartilage zone was wide. The group receiving dry milk plus 0.0001 cc. of viosterol showed the same kind of atypical healing. Of the rats receiving dry milk plus 0.0002 cc. of viosterol, three showed this atypical healing, while the other three rats showed typical healing, *i.e.*, a line of new calcification at the provisional zone.

TABLE III

Effect of Dry Milk on Healing of Rickets

Rat No.	Supplement to diet*	Experimental period	Ca	P	Ca × P	Healing
		days	*mg. per 100 cc.*	*mg. per 100 cc.*		
452–456	Control	0	10.0	2.8	28	−
469–474	0.005 cc. viosterol	10	13.7	4.7	64	++++
475–480	0.25 " cod liver oil	10	13.6	4.0	54	+++
487–492	Dry milk + 0.0002 cc. viosterol	10	11.2	4.5	50	+++
481–486	Dry milk + 0.0001 cc. viosterol	10	12.3	3.9	48	+++
463–468	Dry milk	10	13.1	3.9	51	+++
457, 460, 461	Control	10		2.7		−

* These quantities of viosterol and of cod liver oil were added to 100 gm. of the Steenbock diet. The dry milk was fed so that each rat received the equivalent of 10 cc. of reconstituted milk daily.

DISCUSSION

The results obtained here are consistent with all of our previous experiences: when the Ca × P product was below 30, the calcification process was in abeyance; when the product was above 40, the calcification process was active. These experiments are thus in agreement with our previous findings (4) that when for any reason in active rickets the Ca × P product rises above 40, calcification is resumed within a few hours.

When the amount of added viosterol was sufficient to raise the product above the ricketic level, calcification was obtained; when the amount was inadequate to induce healing, it was inadequate to raise the Ca × P product above the ricketic level.

In the experiments with dry milk it was found that, even without the addition of viosterol, the Ca × P product was raised to 51; with the rise in the product, calcification had been resumed. These results are in apparent contradiction to those obtained by Hess and his collaborators.   On the other hand, it is not impossible that the actual results obtained are in agreement; the apparent disagreement may be due to differences in experimental technique employed in evaluating the results.

Thus the presence of healing in Hess' laboratory is determined[3] by radiographic picture and by histologic examination of decalcified and stained bone sections.   We have found that, in the early stages of healing, new calcification demonstrated to be present by staining of the undecalcified section with $AgNO_3$ may be undetectable on the x-ray picture and on the stained sections of the decalcified bone.   It may be, therefore, that healing may have been present in some cases in which no healing was noted by Hess' technique.

In the second place a negative line test does not necessarily mean that no healing has occurred.   With the atypical healing obtained on addition to the diet of non-irradiated dry milk, alone or with very small amounts of viosterol, new bone material was deposited within the metaphysis *but no line was obtained at the provisional zone*.   In the analogous experiments of Hess *et al.* (1) a negative line test was taken to mean "absolute lack of healing" (*cf.* the last column of their Table I).

In the third place, the values obtained for inorganic serum phosphate may be higher in hemolyzed sera than in unhemolyzed sera.   With our technique, hemolysis rarely occurred; the few cases in which slight hemolysis did occur are so identified.   In the rare instances in which marked hemolysis occurred, the serum was discarded without analysis.   In Hess' laboratory, hemolysis was the rule rather than the exception.[4]

Finally, pooling of the blood samples from rats indiscriminately

[3] Personal communication.
[4] Personal communication.

will obscure the situation, as was demonstrated previously (4).
In order to obtain unambiguous results, the blood samples should
be pooled *after* the histological examination is made.   When this
is done, the confusion resulting from the pooling of blood of rats
showing no healing with that of rats showing varying degrees of
healing, is avoided.   For example, in Table II of Hess *et al.* (1)
entitled "Lack of relationship between healing of rickets and
inorganic phosphorus of blood" the analyses for serum Ca and P
were performed on indiscriminately pooled blood samples; blood
from rats showing healing was mixed with blood from rats showing
none.

Another unusual finding reported by Hess *et al.* is the occurrence
of the peculiarly low values of 0.39, 0.19, and 0.15 mg. per cent for
inorganic serum phosphorus (see (1), Table I).   So far as we are
aware no other investigators have obtained such low values,
even in the severest types of rickets.   The question arises as to
whether analytical errors were responsible for such strangely low
results.

· According to this same table, addition of cod liver oil plus non-
irradiated dry milk resulted in most cases in *lower* serum P values
than addition of the same amount of non-irradiated dry milk alone.
It was also reported that irradiated dry milk gave serum P values
which were *lower* than those given by non-irradiated dry milk;
yet healing was obtained with the former and not with the latter.

These discrepancies may be due to the unreliability of the
criteria employed in diagnosing the presence or absence of healing.
Thus, it is widely assumed that *decalcified* bone, stained with
hematoxylin, gives reliable information regarding the presence
and distribution of newly deposited bone salts.   But Cameron (5),
in an admirable study, has recently shown that this method is
open to serious criticism and may lead to grave errors.   In the
light of Cameron's important investigation it would seem that
conclusions drawn from histological examination of decalcified
bones should be regarded with considerable caution.   This bears
out the earlier findings of Eaves (6).

Some of the discrepancies may have been due to the reliance
placed upon the radiograms.   The very early stages of healing
which escape detection by the x-ray method are clearly demon-
strable on the $AgNO_3$-stained sections of the undecalcified bones.
Marshall and Knudson (7) have made similar observations; they

noted instances in which the histological examination "showed perceptible healing which was not visible by the radiograph." Furthermore, even comparatively large amounts of fresh calcification may escape notice on the x-ray plate if the healing be atypical. Healing sometimes first occurs within the metaphysis in isolated areas, or in *vertical* lines in the deep tongues of cartilage. Bills, Honeywell, Wirick, and Nussmeier (8) also mention some of the less usual types of healing. When no line is seen at the provisional zone on the x-ray plate, it does not necessarily mean that calcification has not been resumed, for the newly deposited bone salts may have been deposited in one of the less usual areas or in one of the less usual patterns.

Attention is therefore called to the fundamental importance, in such studies in rickets, of evaluating the validity of the techniques used in examining the experimental material.

SUMMARY

1. Diets containing 0.001 cc. and 0.0005 cc. of viosterol 100 D per 100 gm. of ricketogenic ration raised the Ca × P product above the ricketic level; the rise in the Ca × P product was accompanied by healing of the rickets.

2. When inadequate amounts of viosterol were added to the diet, healing was not obtained; the Ca × P products did not, in such cases, rise above the ricketic level.

3. Addition of dry milk, non-irradiated, to the ricketogenic diet resulted in atypical healing of the rickets.

4. Whenever the Ca × P product was raised above the ricketic level, healing was noted.

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THE POSSIBLE SIGNIFICANCE OF l-XYLOKETOSE (URINE PENTOSE) IN NORMAL METABOLISM*†

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Our knowledge of the nature of the metabolic disturbance in pentosuria is very slight. Experiments have failed to show any connection with carbohydrate metabolism, but have rather indicated a relation to that of protein (1, 2). Moreover, if pentosuria were due to a deranged glucose catabolism, one would expect to find some severe types, comparable to diabetes in their effect on the individual. If such severe cases existed, they would almost certainly have been observed. Since they have not been detected and since the amount of pentose excreted is never great, it is probable that pentosuria has nothing to do with ordinary carbohydrate metabolism.

Unfortunately, in none of the patients with pentosuria, upon whom metabolism experiments have been performed, was there any satisfactory identification of the nature of the pentose excreted. Since it would appear that there are two kinds of pentosuria, in one of which dl-arabinose is excreted and in the other l-xyloketose, it would be of great value to repeat these experiments upon individuals in whom the nature of the sugar had been established.

In a previous publication, the author (3) has reported on the nature of the sugar in four cases of pentosuria, in all of which the sugar was found to be l-xyloketose. Unfortunately, not one of

these four could be induced to proceed with a metabolism experiment. However, it appeared to be of interest to determine to what extent this sugar could be used by a normal animal. If it were not utilized, or to no greater extent than are xylose or arabinose, it would be quite obvious that *l*-xyloketose was the product of an abnormal metabolism and bore no relation to normal catabolism of either carbohydrate or protein. But, if it

TABLE I

Effect of l-Xyloketose and of d-Xylose upon Sugar Excretion

Dog 81, female, weight 5.6 kilos, fed a mixture of 100 gm. of hashed beef heart, 16 gm. of cracker meal, 16 cc. of maize oil, 6 gm. of bone ash, and 200 cc. of water.

Date	Urine		Remarks
	Nitrogen	Sugar*	
1988	*gm.*	*gm.*	
June 11	3.19	0.092	
" 12	2.37	0.070	
" 13	3.16	0.303	*l*-Xyloketose equivalent to 3.80 gm. glucose in 5 doses
" 14	2.65	0.097	
" 15	2.62	0.077	
" 16	2.57	0.079	
" 17	2.67	0.100	
" 18	3.18	2.35	3.31 gm. *d*-xylose equivalent to 3.82 gm. glucose in 4 doses
" 19	2.31	0.070	
" 20	3.06	0.086	
" 21	2.84	0.094	3.40 gm. glucose in 5 doses

* Calculated as glucose.

were utilized, it might be a normal intermediary catabolite. Accordingly, two experiments were performed upon dogs.

The diets employed are indicated in Tables I and II, as are the results of the analyses of the urine samples for nitrogen and sugar. Sumner's method (4) was used for the latter purpose. The periods began and ended at 9 a.m. On the days indicated, the dogs were injected, subcutaneously, with a solution of *l*-xyloketose, prepared from the *p*-bromophenylhydrazone. The

"glucose equivalent" of the sugar injected is indicated in Tables
I and II.   In other experiments (3), it was found to be 1.25 times
the amount of pentose calculated to be present.   In subsequent
control experiments upon the same animals, d-xylose was injected.

TABLE II

Effect of l-Xyloketose and of d-Xylose upon Sugar Excretion

Dog 8, male, weight 10.7 kilos, fed a mixture of 110 gm. of hashed meat,
30 gm. of cracker meal, 30 cc. of maize oil, 10 gm. of bone ash, and 450 cc. of
water.

Date	Urine		Remarks
	Nitrogen	Sugar*	
1930	*gm.*	*gm.*	
June 1	3.83	0.211	
" 2	2.12	0.115	
" 3	2.95	0.157	
" 4	1.90	0.591	l-Xyloketose equivalent to 7.7 gm. glucose in 5 doses
" 5†	3.63	0.836	
	3.03	0.108	
" 6	4.61	0.167	
" 7	3.07	0.123	
" 8	4.41	0.208	
" 9	2.13	0.119	
" 10	2.43	0.152	
" 11	4.31	5.90	7.54 gm. d-xylose, equivalent to 8.72 gm. glucose in 5 doses
" 12	3.15	0.268	
" 13	2.28	0.173	
" 14	5.93	0.464	
" 15			

* Calculated as glucose.
† Voided before 4.30 p.m.

The results are quite obvious.   Whereas, 60 to 65 per cent of
the injected xylose appeared in the urine, only 6 per cent (in
one experiment) and 14 per cent (in the second) of the xyloketose
was recovered.   It is quite clear, therefore, that l-xyloketose
can be further metabolized in the body.   Whether or not it plays
a part in normal metabolism remains to be determined.   A curious

result, not readily explained, was the increase in nitrogen excretion observed after the injection of both xylose and of *l*-xyloketose. This was more marked in the second experiment than in the first.

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IDENTIFICATION OF REDUCING SUBSTANCES IN NEPHRITIC URINE

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In a study of the excretion of reducing substances in nephritis Hawkins, MacKay, and Van Slyke (1) found that the fermentable reducing substances were frequently increased in all types of the disease, and that in some of the degenerative cases the excretion amounted to a gross melituria.

The present work is an attempt to identify these fermentable reducing substances.

EXPERIMENTAL

Analytical Methods—The total and non-fermentable reducing substances in the urine were determined by the gasometric methods of Van Slyke and Hawkins (2) and recorded in terms of glucose.

Diurnal Excretion of Reducing Substances—In the two cases studied the daily amount of fermentable and non-fermentable reducing substances excreted was determined on several successive days. The diurnal excretion was determined on 3 hour specimens during a 24 hour period. The results are recorded in Figs. 1 and 2. The concentration of total reducing substances in the urine of Case I varied mostly between 0.84 and 0.96 per cent, with 0.12 to 0.15 per cent non-fermentable. In Case II the total reducing substances were about 0.20 per cent, of which 0.06 per cent was non-fermentable.

Decolorization and Removal of Protein from Urine—The urine samples used were slightly acid to litmus. Alcohol was added to make a 20 per cent alcoholic solution, since in such a solution the amount of reducing substances adsorbed on charcoal during decolorization is reduced from 20 per cent of the total to 3 per cent

(3, 4). To every liter of this solution about 10 gm. of norit were added; the mixture was shaken 15 minutes and filtered. The proteins were removed by adding to the filtrate twice its volume of 95 per cent alcohol and filtering. The filtrate was concentrated under diminished pressure.
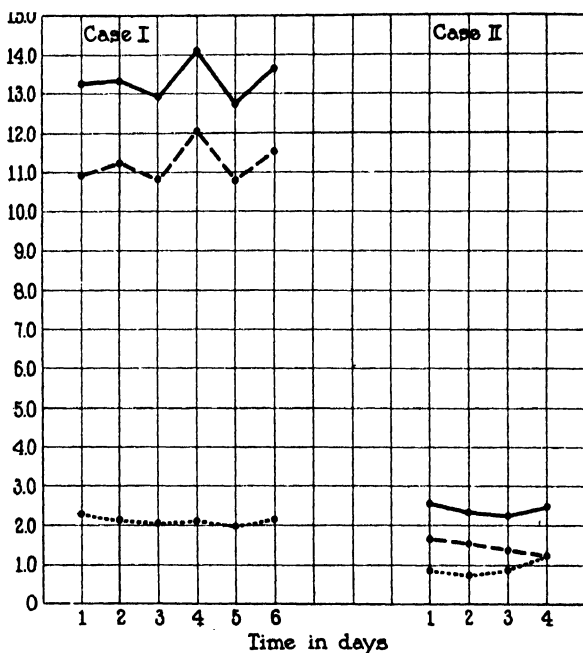
Fig. 1. Daily excretion of fermentable and non-fermentable reducing substances in two cases of nephritis. ————, total reducing substances excreted. — — —, fermentable reducing substance excreted. ...., non-fermentable reducing substances excreted.

An *osazone* was formed from the urine concentrated as described above by adding 2 parts of phenylhydrazine hydrochloride freshly recrystallized from 95 per cent alcohol and 3 parts of sodium acetate to 1 part of reducing substance calculated as glucose. The mixture was allowed to stand 15 minutes at room temperature in order to note any tendency to hydrazone formation, then placed in a boiling water bath for 2 hours. The osazones which had formed in the hot solution were filtered off immediately with suc-

tion, washed with hot water, then with 95 per cent alcohol, and with methyl alcohol. The crystals thus obtained were light yellow and were recrystallized once from 70 per cent alcohol. The melting point was estimated by raising the temperature about 1° per 10 seconds and correcting. The mixed melting point with glucosazone was also taken. The glucosazone was prepared as described above from pure glucose and recrystallized once from abso-
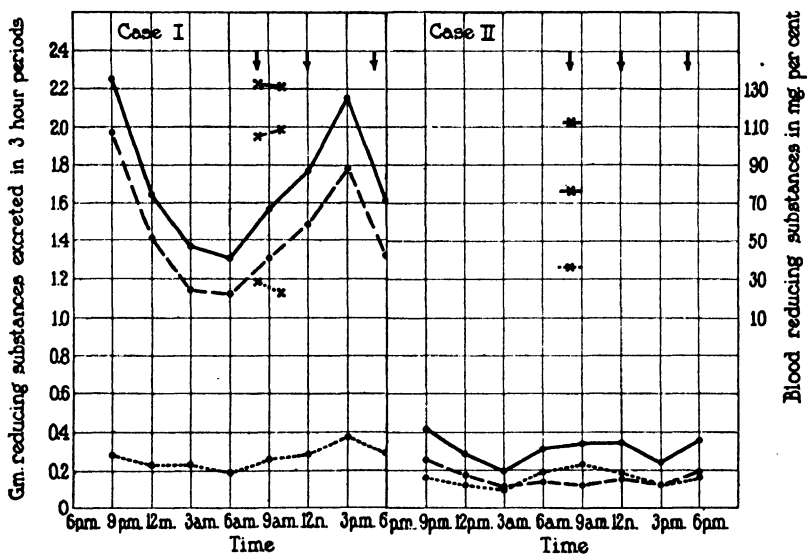
FIG. 2. 3 hourly excretion of fermentable and non-fermentable reducing substances over a period of 24 hours. .————., total reducing substances excreted. .— — — —., fermentable reducing substance excreted. ...., non-fermentable reducing substances excreted. ×————×, total reducing substances in blood. ×— — — —×, fermentable reducing substance in blood. ×........×, non-fermentable reducing substances in blood. Arrow designates time of meals.

lute alcohol. The mutarotation was determined by dissolving 0.0200 gm. of the osazone in 5 cc. of a mixture of 3 parts of dry pyridine to 2 parts of absolute alcohol. The results recorded in Table I show that the osazones from the urine differed from glucosazone only to the extent that might be expected from small amounts of impurities in the osazones from the urine.

Osazones Soluble in Hot Aqueous Solution. Urine 1—After removal of the large crystals of osazones which constituted the first

crop from the hot solution as described above, a crop of small, darker crystals was obtained on cooling. Similar crystals were obtained from the dark alcohol washings of the first osazone. The melting points of these osazones varied greatly and were indefinite (from 171–194°). The mutarotation in a mixture of pyridine and absolute alcohol was indicated by the following specific rotation.

Within 12 minutes.................................. $[\alpha]_D$ − 46.5

Bromination—A portion of the urine prepared and concentrated as described above was treated with bromine for 48 hours with

TABLE I

Properties of Osazones Insoluble in Hot Aqueous Solution Obtained from Urine in Nephritis

Origin of osazone	Melting point (corrected)	Mixed melting point with glucosazone (corrected)	Mutarotation in pyridine-alcohol	
			Within 15 min. $[\alpha]_D$	After 24 hrs. $[\alpha]_D$
	°C.	°C.		
Glucose.....................	207–208		−70.6	−20.3
Urine 1.....................	204–205	203–204	−66.6	−20.5
" 2.....................	202	202	−74.3	−19.8

occasional shaking. The amount of fermentable and non-fermentable reducing substances was estimated before and after treatment with bromine. The results are recorded in Table II. The fermentable reducing substance behaved like glucose in being completely destroyed by bromination. The non-fermentable reducing substances were unaffected by bromination. Everett and Sheppard (5) found that the reducing substance of normal urine is not destroyed by bromination.

Oxidation with Nitric Acid—A portion of the urine prepared as described above and containing about 3.5 gm. of reducing substances calculated as glucose was concentrated to about 10 cc. and treated with 2.5 times its volume of a solution containing equal parts by volume of nitric acid and water. After standing overnight the crystals of urea nitrate were filtered off with suction, the

filtrate was treated with more acid and evaporated on a large watch-glass on the water bath, with constant stirring and rubbing. As no mucic acid separated out, evaporation was continued to a paste, which was washed with a few cc. of water into a small beaker, neutralized with an excess of 50 per cent potassium hydroxide, then treated with an excess of glacial acetic acid. After standing in the ice box for several days the crystals were filtered cold, washed with 1 cc. of ice water, recrystallized from hot water, and dried in a vacuum desiccator at 70°. This procedure was essentially the same as that used by Heidelberger and Goebel[1] for the oxidation of aldobionic acid. The potassium acid salt was analyzed for potassium gravimetrically by conversion to potas-

TABLE II

Effect of Bromination on Fermentable and Non-Fermentable Reducing Substances of Urine in Nephritis

Specimen	Treatment	Total reducing substances	Non-fermentable reducing substances	Fermentable reducing substances
		per cent	*per cent*	*per cent*
Urine 1	Before bromination	4.62	0.63	3.99
	After "	0.64	0.65	0
	Amount removed	3.98		3.99
Urine 2	Before bromination	0.43	0.21	0.22
	After "	0.18	0.18	0
	Amount removed	0.25		0.22

sium sulfate. The results check those calculated for the potassium acid salt of saccharic acid.

        Calculated. K 15.75 per cent
        Found.   " 15.78 "   "

Precipitation of Reducing Substances with Copper Sulfate and Calcium Hydroxide—An attempt was made to separate the reducing substances with copper, in the hope of separating glucose from the other constituents. The method developed by Van Slyke (6) for precipitating glucose with copper sulfate and lime was used. For every 125 cc. of Urine 1 concentrated as described above and containing about 1 per cent of reducing substances calculated as

[1] Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, **70**, 613 (1926).

glucose, 50 cc. of a 20 per cent solution of copper sulfate were added, followed by a 10 per cent suspension of calcium hydroxide, which was added with shaking until the reaction was alkaline to litmus. After standing 30 minutes the precipitate was filtered off with suction, suspended in water, and acidified with sulfuric acid. The copper was removed with hydrogen sulfide and the filtrate concentrated under diminished pressure. Both total and non-fermentable reducing substances were estimated before and after hydrolysis with 0.2 N sulfuric acid for 1 hour, and also before and after bromination. Only 42 per cent of the reducing substances was recovered from the precipitate, but non-fermentable as well as

TABLE III

Reducing Substances Precipitated from Urine 1 with Copper Sulfate and Calcium Hydroxide

Treatment	Total		Non-fermentable	Fermentable	Increase or decrease, fermentable	Increase or decrease, non-fermentable
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before precipitation...............	7.46					
After " *...............	3.13	0.735	0.112	0.623		
" " + hydrolysis*.		0.789	0.140	0.649	+0.026	+0.028
" " + bromination*...........................		0.165	0.153	0.012	−0.611	+0.041

* Figures represent material recovered from the copper-lime precipitate, not from its filtrate.

fermentable substances were recovered in practically the same proportions that were originally present in the urine. These results are shown by comparison of Table III with Fig. 1. Hydrolysis of the precipitated fraction showed a slight increase in reducing substances, both for the fermentable and the non-fermentable fraction. Bromination caused a loss of 98 per cent of the fermentable reducing substance, and a slight increase in the non-fermentable fraction; the latter change was probably caused by hydrolysis with hydrobromic acid formed by the bromine in aqueous solution.

Non-Fermentable Reducing Substances. Urine 1—After treatment with norit, removal of proteins, and concentration, the urine was treated with yeast until all fermentable reducing substance

was removed.   The time required was $\frac{1}{2}$ hour.   The fermented
solution was subjected to a series of tests for various carbohydrate
groups and to hydrolysis.   The results are recorded in Table IV.
Osazones were formed as described above both before and after
hydrolysis.   The osazones in this case appeared only slowly in the
cooled solution.   No osazones could be obtained from yeast sim-

TABLE IV

Non-Fermentable Reducing Substances of Urine 1

Reactions		Time of hydrolysis	Reducing substances
		hrs.	*per cent*
Molisch test for carbohydrates	+		
Phloroglucinol test for pentose	−		
Resorcinol test for ketose	−		
Naphthoresorcinol test for glucuronic acid	+		
Hydrolysis with 0.5 N HCl		0	1.28
		3	1.34
		6	1.24
		12	1.20

TABLE V

Properties of Osazone Obtained from Urine 1 after Fermentation with Yeast

Treatment	Melting point (corrected)	Mutarotation	
		Within 20 min. $[\alpha]_D$	After 48 hrs. $[\alpha]_D$
	°C.		
Before hydrolysis............................	186–194	−60.6	−36.4
After 30 min. hydrolysis in 0.2 N H_2SO_4.....	194–203	−35.0	−28.0

ilarly treated.   The properties of the osazones are recorded in
Table V.
The osazones (Table V) were soluble in acetone and had variable
and indefinite melting points.   Both of these properties are char-
acteristic of the product formed by the reaction of phenylhydra-
zine on glucuronic acid, which forms a series of compounds (hy-
drazone, hydrazide, hydrazone hydrazide, osazone hydrazide)
with melting points between 107–217° (7–11).

A further separation of the non-fermentable reducing fraction was attempted through the precipitation of glucuronates with basic lead acetate. The fermented Urine 2 concentrated as described above, was treated first with neutral lead acetate to precipitate phosphates, sulfate, and other impurities which would otherwise be included in the glucuronate precipitate. The glucuronates were then precipitated with basic lead acetate. The

TABLE VI

Effect of Hydrolysis on Reducing Substances Precipitated by Basic Lead Acetate

Treatment	Time of hydrolysis	Reducing substances
	min.	*per cent*
Before hydrolysis.	0	0.57
After "	10	0.69
" "	30	0.64
" "	60	0.66

TABLE VII

Properties of Osazone Obtained from Urine 2 after Fermentation with Yeast and Treatment with Basic Lead Acetate

Age of osazone	Melting point (corrected)	Mixed melting point with glucosazone (corrected)	Mutarotation in pyridine-alcohol	
			Within 15 min. $[\alpha]_D$	After 24 hrs. $[\alpha]_D$
	°C.	*°C.*		
Freshly prepared.	204–205			
After 1 wk.	184–194	184–194	−48.8	−14.4

precipitate, after suspension in water and removal of lead with hydrogen sulfide, reduced Benedict's solution and gave a strong naphthoresorcinol reaction. The precipitation process was repeated. The solution was evaporated to dryness under diminished pressure, taken up in methyl alcohol; insoluble substances were removed by centrifugation, and the alcohol by evaporation under diminished pressure. The complete removal of acetic acid by distillation under diminished pressure was unsuccessful, as the solution became colored and the reducing power decreased.

An aqueous solution of the reducing substances was hydrolyzed with 0.2 N sulfuric acid and analyzed at intervals for 1 hour. The results are recorded in Table VI.

The formation of a combination with parabromophenylhydrazine was unsuccessful, probably because of insufficient material. A brown sticky mass was obtained instead of crystals. An attempt to form a cinchonine combination was likewise unsuccessful.

The filtrate from the first basic lead acetate precipitation was acidified, treated with hydrogen sulfide to remove the lead, neutralized, and treated with phenylhydrazine. The osazone appeared slowly on cooling, and was recrystallized from 30 per cent alcohol. The osazone decomposed rapidly on standing in a desiccator, so that by the time the mixed melting point was taken the crystals were dark brown and the melting point very indefinite. The melting points and mutarotation are recorded in Table VII.

DISCUSSION

Among the sugars which form osazones with melting points between 200–208° (Table I) are glucose, levulose, mannose, lactose, and maltose. The levorotation of the osazone ruled out maltose, the osazone of which is dextrorotatory. Lactose was ruled out by the fact that no mucic acid was formed on oxidation with nitric acid. Glucose, levulose, and mannose form identical osazones, which therefore have the same melting point, the same mutarotation, and which, when mixed with glucosazone, show no change in melting point. Since the osazones from both urines showed mutarotation in a mixture of pyridine and absolute alcohol similar to that of glucosazone (Table I), and the mixed melting point with glucosazone showed no change from the original melting point, it can be concluded that the sugar is identical with one of these three. Mannose, however, forms a hydrazone on standing at room temperature for 15 minutes with phenylhydrazine. Such a reaction could not be demonstrated with either of the urine samples. The differentiation between glucose and levulose was made by means of bromination. Bromine will oxidize 98 per cent of the aldo groups present in an aqueous solution, but has no effect on the keto groups. Table II shows that all fermentable reducing substance was completely removed from the concentrated urine in both cases; therefore, the fermentable fraction is entirely composed of sugar con-

taining aldo groups.   The non-fermentable fraction was unaffected by bromine.   Since this reaction ruled out levulose, it follows that the similarity of these osazones with glucosazone in melting point, mixed melting point, and mutarotation points to the identification of the fermentable sugar in these two urine samples as glucose.   This identification was further substantiated by the oxidation of the sugar with nitric acid and the formation of a potassium acid salt of the oxidation product, which proved on analysis to be the potassium acid salt of saccharic acid.

The more soluble osazones obtained by slow cooling as a second crop were small in amount and dark in color.   The melting points were indefinite and varied greatly, from 171–194°.   The mutarotation was shown by the change from -46.5 or $[\alpha]_D$ in pyridine-absolute alcohol within 12 minutes to -23.2 after 24 hours. From the mutarotation an impure glucosazone or a mixture of osazones might be suspected.   Since glucosazone is not entirely insoluble in hot water or cold alcohol, it is likely that some glucosazone was present in this fraction.

SUMMARY

The fermentable reducing substance excreted in the urine of two patients with nephritis is identified as glucose.

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THE DETERMINATION OF SODIUM PLUS POTASSIUM AS BENZIDINE SULFATE

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The benzidine method for sulfate estimation of Rosenheim and Drummond (1) was adapted to the determination of total base by Fiske (2). The technique described by Stadie and Ross (3) has been used successfully in this laboratory for the total base of blood serum. However, for such materials as feces, milk, and urine with a large content of calcium, magnesium, and phosphorus, the results obtained are definitely low. The phosphate must be removed so that the base may be present as sulfate. This is accomplished by the addition of iron. Unless special precautions are used, the subsequent removal of iron by ammonia causes losses of calcium and magnesium. In addition, when a large part of the base is present as calcium, the solubility of calcium sulfate presents a special problem. These difficulties, though not pointed out in the original article, are recognized by Stadie and Ross. A technique for the accurate determination of total base in such materials is now being developed in their laboratory.[1] For the determination of sodium and potassium together, the present total base technique would be excellent if *all* the calcium and magnesium were first removed. An adaptation of the method of Stadie and Ross (3) designed to accomplish this end is described below.

Method

To remove the calcium, magnesium, and phosphorus, the procedure is as follows. The sample to be analyzed is ashed in a muffle furnace just under red heat. Urine may be ashed with sulfuric and

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[1] Private communication from Dr. S. L. Wright, Jr.

nitric acids in silica. The ash is dissolved with hydrochloric acid and made to a suitable volume. An aliquot of the ash solution, , which contains 0.15 milli-equivalent or more of sodium and potassium, is diluted to approximately 50 or 25 cc., depending upon whether the aliquot contains more or less than 15 mg. of calcium. The solution is heated to boiling. From 2 to 5 cc. of saturated ammonium oxalate (the amount dependent upon the calcium content) are added slowly and the solution made very alkaline with 3 to 5 cc. of concentrated ammonia. If the aliquot does not contain sufficient phosphorus to precipitate the magnesium completely, 5 cc. of 0.1 M $(NH_4)_3PO_4$ are added. The determination is allowed to stand at least 2 hours—preferably overnight. The supernatant liquid is filtered from the precipitate through either No. 40 Whatman 9 cm. filter paper or through a Gooch crucible, and washed, once by decantation and six to eight times with 2 per cent ammonia water. The excess ammonia is boiled off and a sufficient amount of ferric alum added to precipitate all the phosphate present. 1 cc. of 1.0 N ferric ammonium sulfate (321 gm. of $Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$ made to 1000 cc.) completely precipitates 0.2 milli-equivalent or 2 mg. of phosphorus. Next, concentrated ammonia is added slowly to precipitate all the iron present. In order to avoid an excess of ammonia, the number of drops of concentrated ammonia which will just precipitate all the iron in 1 cc. of the reagent is determined. About 8 or 10 drops are usually required. The sample is made up to 100 cc. or 50 cc. when more or less than 3 cc. of alum are used, respectively.

From this point the technique described by Stadie and Ross (3) is followed. An aliquot, which contains 0.15 milli-equivalent of sodium and potassium, is placed in a 50 cc. silica crucible. 10 to 15 drops of concentrated sulfuric acid and 2 cc. of concentrated nitric acid are added and the sample is evaporated to dryness (with care against spitting). Before the evaporation is complete, more nitric acid may be needed. The ash is heated at first gently and finally to red heat for 10 minutes. When cool, the ash is dissolved in water, transferred to a graduated tube, and made to 15 cc.; 2 cc. of benzidine reagent[2] are added and the precipitate

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[2] 4 gm. of benzidine, 45 cc. of N HCl, made to 250 cc. and filtered. 2 cc. of the reagent are standardized by titration with 0.02 N NaOH.

allowed to stand for 3 minutes. The solution is filtered through No. 40 Whatman 5.5 cm. filter paper, and 15 cc. of the filtrate titrated with 0.02 N NaOH with phenol red as an indicator. The milli-equivalents of combined sodium and potassium in the aliquot = 0.02 × (titer of 2.00 cc. of benzidine HCl − $\frac{1}{15}$ titer of 15 cc. of filtrate).

The potassium may be determined on the original ash according to the method of Shohl and Bennett (4). The difference between

TABLE I

Sodium Plus Potassium Values in Known Salt Solutions, Feces, and Milk, As Determined by Authors' Method

Figures are in terms of the sample actually taken.

Sample	Na and K				Error
	Present	Added	Total	Recovered	
	m.-eq.	*m.-eq.*	*m.-eq.*	*m.-eq.*	*per cent*
Salt solution	0.141*		0.141	0.142	+0.6
				0.142	+0.6
				0.143	+1.4
				0.140	−0.6
Salt solution	0.310†	0.143‡	0.453	0.452	±0
	0.310†	0.286‡	0.596	0.590	−1.0
Feces ashing	0.097†	0.119§	0.206	0.210	+2.0
	0.097†	0.143‡	0.240	0.238	−0.9
	0.194†	0.143‡	0.337	0.340	+0.9
Powdered milk	0.205†	0.143‡	0.348	0.342	−1.7

* All potassium, by analysis.
† Analysis by authors' method.
‡ NaCl addition, standardized solution.
§ K₂SO₄ addition, standardized solution.

the milli-equivalents of total sodium and potassium and the milli-equivalents of potassium gives the sodium value.

Results

Typical results obtained with this method are given in Table I. The salt solution taken was similar in mineral content to that of feces of infants in which the sodium and potassium constitute only

14 per cent of the base. Each cc. contained 13.2 mg. (0.66 milli-equivalent) of calcium, 6.3 mg. (0.61 milli-equivalent) of phosphorus, 2.4 mg. (0.20 milli-equivalent) of magnesium, and 5.11 mg. (0.141 milli-equivalent) of potassium. Duplicate determinations gave values well within the error allowed by Stadie and Ross (3) (±2 per cent). To this solution was added sodium chloride in amount equivalent to 50 and 100 per cent of the potassium present. Recoveries were within ± 2 per cent of the calculated figures.

Splendid duplicates were obtained on the ash of feces and milk. Various known amounts of sodium and potassium were added to the samples. Both sodium and potassium were recovered within the accuracy of the method.

TABLE II

Comparison of Stadie and Ross' Method for Total Base and the Authors'
Method for Sodium and Potassium in Urine

Figures are in terms of the sample actually taken.

Sample No.	Na and K authors' method	Ca*	Mg.†	Total base calculated	Stadie and Ross method
	m.-eq.	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*
I	0.123	0.005	0.012	0.140	0.124
II	0.127	0.005	0.014	0.146	0.123

* CaC_2O_4 precipitation, titration with $KMnO_4$.
† $MgNH_4PO_4$ precipitation, colorimetric determination.

The results obtained for urine of infants by our method were compared with those obtained by the total base method of Stadie and Ross (3) (Table II). Both values are essentially the same. The agreement shows that all of the calcium and magnesium were lost under the conditions of the total base determination as carried out by us.

DISCUSSION

Calcium oxalate may occlude some sodium and potassium unless precipitated in a comparatively large volume. 50 cc. are adequate for the amount of calcium usually found in feces and milk. The volume occupied by the ferric hydroxide gives a positive error. 1 cc. of the ferric alum reagent yielded a precipitate of hydroxide which occupied a space equal to 0.2 cc. No allowance need be

made in the calculation for this unless the volume of the precipitate is more than 1 per cent of the total volume. It is important to keep the ash at red heat for *only 10 minutes,* as prolonged heating at redness converts the sulfates into another compound.[3] The conditions necessary for the precipitation and determination of the benzidine sulfate have been discussed fully by Stadie and Ross (3).

The method here described removes the interfering anions and cations with two precipitations: (*a*) CaC_2O_4 and $MgNH_4PO_4$, (*b*) $FePO_4$ and $Fe(OH)_3$. The gravimetric determination (5) requires five separate precipitations and washings. The advantages of the procedure are evident: much less material is required, less opportunity for loss is afforded, and the time taken for the whole procedure is greatly reduced. The accuracy is sufficient for the demands of most biological problems.

SUMMARY

An adaptation of the total base method of Stadie and Ross is described for the determination of sodium and potassium. It is comparatively rapid, applicable for small quantities of material, and accurate ± 2 per cent.

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[3] For this information we are indebted to Dr. S. L. Wright, Jr.

HEXOSEMONOPHOSPHATE (ROBISON)

NATURAL AND SYNTHETIC

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In a previous communication[1] we described a synthetic glucose-phosphate, prepared through monoacetone glucose, and concluded that its structure was glucose-6-phosphate as its osazone was identical with that of the Harden-Young and Neuberg esters. At the same time we pointed out that in other properties, including enzymic fermentation, it was quite like the Robison ester. The assignment of the structure of glucose-6-phosphate to the Robison ester was supported by all the available data with the single exception of the osazone, that of the Robison ester having been described[2] as melting at 139° while that of the Harden-Young and Neuberg[3] esters melted at 151–152°. We pointed out that to us the only apparent alternatives were either that the osazone described by Robison was impure and consequently had a low melting point, or else that the two glucosides which can be formed from the Robison ester are not 1, 4 and 1, 5 as is the case of ordinary sugars.

In order to establish conclusively the fact that positions 4 and 5 are unoccupied in the Robison ester, we have undertaken a study of the lactone formation of the aldonic acid derived from it. At the same time we prepared the aldonic acids from our synthetic glucose-6-phosphate and from the synthetic glucose-3-phosphate. All three acids exhibit the formation of two lactones so that posi-

[1] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **89,** 479 (1930).

[2] Robison, R., *Biochem. J.*, **16,** 809 (1922).

[3] von Lebedev, A., *Biochem. Z.*, **28,** 213 (1910). Young, W. J., *Biochem. Z.*, **32,** 177 (1911).

tions 4 and 5 are not occupied in any of them (this was previously shown[1] for the glucose-3-phosphate), substantiating the results with the glucosides. Moreover, the Robison ester behaves in all respects like the synthetic 6-phosphate and is quite different from the 3-phosphate, confirming the assignment of the structure of glucose-6-phosphate to the Robison ester.

This leaves unanswered only the matter of the melting points of the osazone. As previously mentioned, we were able to obtain only the osazone of the Harden-Young ester when we used an impure preparation of the Robison ester. The experiment has just now been repeated by Robison and King[4] on a truly pure preparation of the Robison ester and they likewise find that the osazone is identical with that from the Harden-Young ester and melts at 154–154.5° instead of 139° as originally reported by Robison.

Thus all results are in harmony with the assignment of the structure of glucose-6-phosphate to the Robison ester.

The synthesis of the Robison ester reported in the earlier paper may be useful when this ester is required for biological studies; a possibly easier synthesis will be described later.

<div align="center">EXPERIMENTAL</div>

A. Phosphate from Monoacetone Glucose—Recrystallized mono-acetone glucose was phosphorylated as previously described[1] and the acetone group was hydrolyzed off as before with hydrochloric acid. The chloride was removed with freshly prepared silver sulfate, the residual silver was precipitated with hydrogen sulfide, and the excess hydrogen sulfide was removed by aeration. A warm saturated barium hydroxide solution was added until the mixture was just alkaline to phenolphthalein, and it was then centrifuged. The solution was concentrated under reduced pressure to about half volume, filtered with charcoal, and then further concentrated. The barium salt was precipitated with an equal volume of 95 per cent alcohol, washed with 50 per cent alcohol, redissolved, and reprecipitated in the usual way. The dried salt was dissolved in water and the barium was quantitatively removed with sulfuric acid. After centrifuging off the barium sulfate, a methyl alcohol solution of brucine was added to pH 7.0 to 7.2.

[4] Robison, R., and King, E. J., *J. Soc. Chem. Ind.*, *Chem. and Ind.*, **50**, 156 (1931).

After filtering the mixture with charcoal and concentrating to a small volume, 3 volumes of acetone were added. On scratching and standing, the product crystallized. It was filtered off, washed with 75 per cent acetone and then acetone, and was air-dried. It was recrystallized, but with difficulty, by dissolving in hot 90 per cent methyl alcohol and then adding 3 volumes of acetone.

Material crystallized three times had the following analysis.

6.340 mg. substance: 0.304 cc. N (755 mm. and 26.5°).
10.450 "   "   : 22.555 mg. ammonium phosphomolybdate (Pregl).
$C_{52}H_{65}O_{17}N_4P$. Calculated. N 5.34, P 2.96
                 Found.   " 5.42, " 3.13

and its rotation was

$$[\alpha]_D^{25} = \frac{-0.83° \times 100}{1 \times 5.0} = -16.6° \text{ (in water)}$$

$$[\alpha]_D^{25} = \frac{-0.78° \times 100}{1 \times 5.0} = -15.6° \text{ (in pyridine-water, 1:1 by volume)}$$

Some of this material was dissolved in water, and a barium hydroxide solution was added until the mixture was just alkaline to phenolphthalein. The brucine was filtered off and washed with water, and to the combined filtrate and washings a little more barium hydroxide solution was added to restore the pH to about 8.5. The solution was concentrated to a small volume, filtered with charcoal, and precipitated with an equal volume of 95 per cent alcohol. The subsequent washing, solution, reprecipitation, and drying were done in the usual manner.

The analysis corresponded to a barium hexosemonophosphate.

0.0948 gm. substance: 0.0536 gm. $BaSO_4$.
4.110 mg.   "   : 23.760 mg. ammonium phosphomolybdate (Pregl).
$C_6H_{11}O_9PBa$. Calculated. Ba 34.74, P 7.84
                 Found.   " 33.27, " 8.39

and its rotation was

$$[\alpha]_D^{25} = \frac{+1.57° \times 100}{2 \times 4.74} = +16.6° \text{ (in water)}$$

From 2.0 gm. of this material the barium was quantitatively removed with sulfuric acid and the osazone was prepared as previously described. The analysis of the purified osazone was as follows:

4.000 mg. substance: 0.533 cc. N (753 mm. and 26°).
3.635 mg.   "   : 13.930 mg. ammonium phosphomolybdate (Pregl).
   $C_{24}H_{31}O_7N_6P$. Calculated. N 15.38, P 5.68
                        Found.   " 15.07, " 5.56

It melted at 151–152° as did a mixture with the osazone from the Harden-Young diphosphate. Its rotation, in pyridine-absolute alcohol (2:3 by volume), was −43.3° after 15 minutes and

$$[\alpha]_D^{25} = \frac{-\ 0.17° \times 100}{0.5 \times 1.2} = -\ 28.3° \text{ (at equilibrium)}$$

A portion of the barium salt was oxidized to the aldonic acid with barium hypoiodite. 12 gm. of the dry barium salt were dissolved in 60 cc. of water and added to a solution of 7.8 gm. of iodine and 15 gm. of barium iodide in 30 cc. of water. The mixture was stirred with a mechanical stirrer and 240 cc. of a 0.5 N barium hydroxide solution were run in through a dropping funnel over a period of 20 minutes. The mixture was stirred for an additional 15 minutes, made acid with sulfuric acid, and sulfur dioxide was passed in until the iodine was all reduced. Barium hydroxide was added to pH about 8.5 and the precipitate was centrifuged off and washed with warm water until the washes gave a negative test for halides. The precipitate was then suspended in water, stirred mechanically, and sulfuric acid was added in small portions until the barium was all removed. Barium hydroxide solution was cautiously added until the solution contained neither sulfate nor barium, and the mixture was then centrifuged. To the solution a methyl alcohol solution of brucine was added to pH 7.0 to 7.2 and the mixture was filtered with charcoal. It was concentrated under reduced pressure to a volume of about 125 cc. and ⅕ volume of methyl alcohol was added. On standing overnight at 10–15°, the material crystallized in beautiful needles. Further crops were obtained by cooling the mixture and finally by concentrating and cooling. The combined material was twice recrystallized from 50 per cent (by volume) methyl alcohol.

The analysis corresponded to a tribrucine salt of a phospho-aldonic acid.

6.689 mg. substance: 0.328 cc. N (7.56 mm. and 25°).
4.963 "   "   : 7.921 mg. ammonium phosphomolybdate (Pregl).
$C_{75}H_{90}O_{22}N_6P$. Calculated.   N 5.77, P 2.13
                        Found.   " 5.59, " 2.36

and its rotation was

$$[\alpha]_D^{25} = \frac{-1.16° \times 100}{2 \times 1.94} = -29.9° \text{ (in water; dissolved by warming and then cooled)}$$

$$[\alpha]_D^{25} = \frac{-2.58° \times 100}{2 \times 4.84} = -26.7° \text{ (in pyridine-water, 1:1 by volume)}$$

The product was dissolved in warm water and cooled, and sodium hydroxide solution was added, very cautiously, to pH 8.0 to 8.2. The brucine was filtered off and washed with water, and the pH of the combined filtrate and washings was readjusted to 8.0 with more sodium hydroxide. The solution was concentrated under reduced pressure to a small volume, filtered with charcoal, and further concentrated. The material was placed in a desiccator until it became syrupy and was then ground several times with absolute alcohol. It was dried over calcium chloride and then to constant weight over phosphorus pentoxide under reduced pressure. The final product was a granular, colorless substance, the phosphorus content of which corresponded to the trisodium salt of a phosphoaldonic acid.

3.890 mg. substance: 24.900 mg. ammonium phosphomolybdate (Pregl).
$C_6H_{10}O_{10}PNa_3$. Calculated.   P 9.07
                        Found.   " 9.29

Its rotation in water was

$$\frac{+0.19° \times 100}{2 \times 5.0} = +1.9°$$

0.25 gm. of this material was dissolved in water, 1.68 cc. cf 1.0 N hydrochloric acid were added (2.3 equivalents per mol), the mixture was diluted to a volume of 5.0 cc., and its rotation was

measured in a 2 dm. tube with sodium (D) light. The readings (at a temperature of 25°) are given in Table I.

The specific rotations were calculated from these data and are

TABLE I

Observed Rotations during Lactone Formation

Glucose-3-phosphate			Glucose-6-phosphate			Robison monophosphate		
Time		Rotation	Time		Rotation	Time		Rotation
hrs.	min.	degrees	hrs.	min.	degrees	hrs.	min.	degrees
0	1¼	−1.63	0	1½	−0.29	0	1¼	−0.30
0	3	−1.56	0	3	−0.20	0	3½	−0.17
0	7	−1.47	0	6	−0.11	0	6	−0.13
0	15	−1.38	0	10	−0.06	0	11	−0.07
0	31	−1.24	0	17	−0.06	0	26	−0.03
1	8	−1.15	0	37	−0.04	1	6	+0.02
2	7	−1.04	1	17	+0.01	4	0	+0.14
4	30	−0.98	3	45	+0.10			
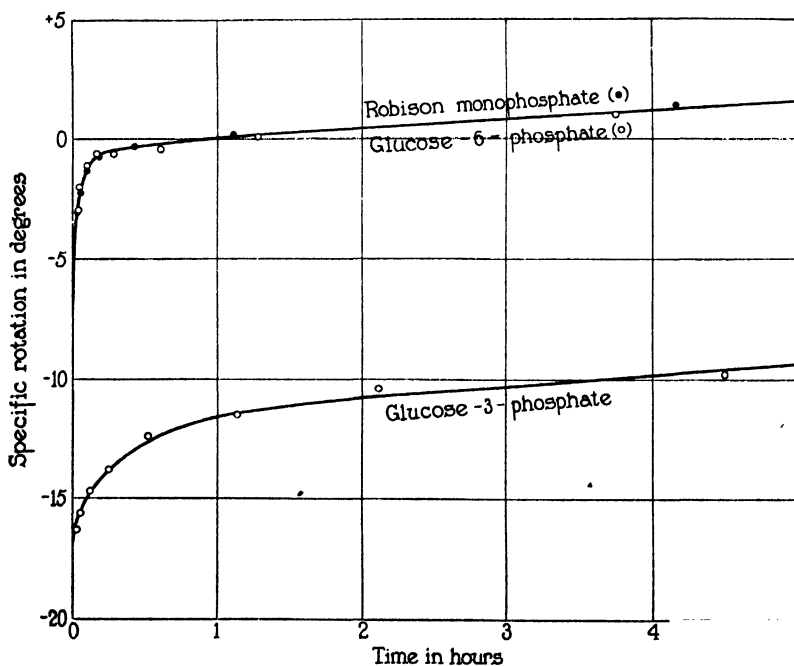
FIG. 1. Observed rotations during lactone formation

given in Fig. 1. The specific rotations are calculated in terms of sodium salt, and not of acid, in order to avoid any question as to the completeness of the reaction between the hydrochloric acid and the salt.

B. Phosphate from Diacetone Glucose—The preparation of this. ester has been previously described, and the properties of the barium and brucine salts have been given. The only additional information which need be given here is the rotation of the brucine salt in pyridine-water which is

$$[\alpha]_D^{25} = \frac{-1.45° \times 100}{2 \times 5.0} = -14.5° \text{ (in pyridine-water, 1:1 by volume)}$$

The osazone, previously described,[5] is an anhydrohexosazone free of phosphorus, the phosphate group having been eliminated during the osazone formation.

The oxidation to the acid with barium hypoiodate, and the conversion to the brucine salt were carried out exactly as described above. The brucine salt behaved a little differently, however, and was twice crystallized from 90 per cent (by volume) methyl alcohol. Its analysis corresponded to a dibrucine salt of a phosphoaldonic acid.

5.400 mg. substance: 0.260 cc. N (747 mm. and 25°).
3.810 " " : 8.050 mg. ammonium phosphomolybdate (Pregl).
$C_{52}H_{65}O_{18}N_4P$. Calculated. N 5.26, P 2.98
Found. " 5.43, " 3.07

Addition of more brucine and recrystallization of the product again led to only a dibrucine salt.

The rotation was

$$[\alpha]_D^{25} = \frac{-0.98° \times 100}{2 \times 2.0} = -24.5° \text{ (in water; dissolved by warming and then cooled)}$$

$$[\alpha]_D^{25} = \frac{-1.55° \times 100}{2 \times 5.0} = -15.5° \text{ (in pyridine-water, 1:1 by volume)}$$

The brucine salt was converted to the sodium salt. Its phosphorus content corresponded to that of a trisodium salt of a phosphoaldonic acid.

[5] Levene, P. A., Raymond, A. L., and Walti, A., *J. Biol. Chem.*, **82**, 191 (1929).

3.885 mg. substance: 23.600 mg. ammonium phosphomolybdate (Pregl).
$C_6H_{10}O_{10}PNa_3$.   Calculated.   P 9.07
Found.   " 8.81

Its rotation was

$$[\alpha]_D^{25} = \frac{+ 0.13° \times 100}{2 \times 5.0} = + 1.3° \text{ (in water)}$$

Hydrochloric acid to the extent of 2.3 equivalents per mol was added to a solution of the sodium salt as in the case of the previous ester. The concentrations were the same and the rotations, measured at 25° in a 2 dm. tube with sodium (D) light, are given in Table I. The specific rotations calculated from these are included in Fig. 1 for comparison. They are almost identical with those previously recorded,[1] the difference being due to the greater acidity used in the present experiments.

C. Robison Monophosphate—This ester was prepared by fermenting a mixture of 4 parts of glucose and 1 part of fructose with yeast juice, phosphate being added every few minutes to maintain a maximum fermentation rate. The phosphate was followed colorimetrically and was kept between 0.01 and 0.04 M. Considerable difficulty was experienced with the procedure as the fermentation and phosphorylation varied greatly from batch to batch and for no assignable reason. Variations of temperature and concentrations gave no consistent results, and finally a large number of preparations were made in an entirely arbitrary fashion. In working up the material, the protein was precipitated with 6 per cent of trichloroacetic acid, and the hexosediphosphate and unchanged phosphate were precipitated from the filtrate by adding barium acetate and then barium hydroxide until alkaline to phenolphthalein. The mixture was filtered, the precipitate washed with water, and the filtrate and washings were combined. Basic lead acetate was added to complete precipitation, the pH being adjusted to about 8 after each addition. The lead salt was well washed with water, suspended in water, and decomposed with hydrogen sulfide. The excess of the gas was removed by thorough aeration, barium hydroxide solution was added to pH about 8.5, and the solution was filtered with charcoal. It was then concentrated under reduced pressure to a small volume and refiltered

with charcoal. The barium salt was precipitated by the addition of an equal volume of 95 per cent alcohol and was washed with 50 per cent and then with 95 per cent alcohol. It was twice suspended in acetone and filtered and was then dried in air at about 40°. The product as thus prepared, contained, according to a Willstätter hypoiodate titration, about 50 per cent of hexosemonophosphate.

It was oxidized with barium hypoiodate exactly as in the case of the synthetic esters except that the amount of ester was changed to correspond to its reduced aldose content, approximately twice as much ester being used for the same quantities of the other reagents. The isolation of the barium salt and the conversion to the brucine salt were carried out exactly as in the other cases. The properties of the brucine salt were similar to those of the product from the monoacetone and the recrystallizations were performed similarly.

The analysis of the product corresponded to that of a tribrucine salt of a phosphoaldonic acid.

6.780 mg. substance: 0.344 cc. N (764 mm. and 24°).
5.890 "   "   : 8.920 mg. ammonium phosphomolybdate (Pregl).
$C_{75}H_{90}O_{22}N_6P$.   Calculated.   N 5.77, P 2.13
                            Found.   " 5.86, " 2.21

The rotation was

$$[\alpha]_D^{25} = \frac{-1.22° \times 100}{2 \times 1.975} = -30.9° \text{ (in water; dissolved by warming and then cooled)}$$

$$[\alpha]_D^{25} = \frac{-2.74° \times 100}{2 \times 4.94} = -27.8° \text{ (in pyridine-water, 1:1 by volume)}$$

This material was converted to the sodium salt as before, the analysis of this substance corresponding to that of a trisodium salt of a phosphoaldonic acid.

4.707 mg. substance: 28.820 mg. ammonium phosphomolybdate (Pregl).
$C_6H_{10}O_{10}PNa_3$.   Calculated.   P 9.07.   Found.   P 8.89.

The rotation in water was

$$[\alpha]_D^{25} = \frac{+0.06° \times 100}{2 \times 5.0} + 0.6°$$

Hydrochloric acid to the extent of 2.3 equivalents per mol was added to a solution of the sodium salt as in the case of the previous esters. The concentrations were the same, and the rotations, measured at 25° in a 2 dm. tube with sodium (D) light, are given in Table I.   The specific rotations calculated from these data are included in Fig. 1.

CONFIGURATIONAL RELATIONSHIP OF HYDROCARBONS

II. OPTICAL ROTATIONS OF HYDROCARBONS OF THE NORMAL SERIES

BY P. A. LEVENE AND R. E. MARKER

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

The simplest group of substances studied with regard to relationships of structure and of optical activity is that of the secondary aliphatic carbinols. In these substances only two variable radicles are present as can be seen from figure (I).

$$
\begin{array}{cc}
R_1 & CH_3 \\
| & | \\
HCOH & HC-C_2H_5 \\
| & | \\
R_2 & C_3H_7\ (n) \\
\\
I & II
\end{array}
$$
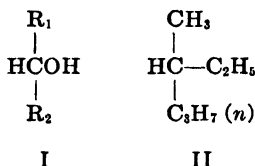
The simplest optically active hydrocarbon is a trisubstituted methane, namely methylethyl-*n*-propylmethane (II). From this hydrocarbon three different homologous series can be formed, depending upon whether the methyl, ethyl, or propyl group is substituted by a higher homologous radicle. In the present study only two of these series were investigated, namely the one derived by the progressive substitution of the group ethyl and the other derived by the progressive substitution of the group propyl. Also the series from the hydrocarbon (III) by progressive substitution of the group ethyl was studied.

$$
\begin{array}{c}
CH_3 \\
| \\
HC-C_2H_5 \\
| \\
C_4H_9\ (n) \\
\\
III
\end{array}
$$

761
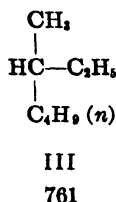
It is evident that it was necessary to establish the configurational relationships of these three groups of hydrocarbons before they could be made use of for the purpose of discussing the relationship of chemical structure to optical activity.   The task was no longer difficult inasmuch as the configurational relationship of disubstituted propionic acids has been established and inasmuch as the hydrocarbons can be derived directly from these acids.

In regard to the acids it will be recalled, in this place, that the dextro-1,1-methylethylpropionic acid (3) was correlated to levo-1,1-methylpropylpropionic acid (3) and that these two configurationally related acids lead to two enantiomorphous hydrocarbons.

$$
\begin{array}{ccc}
C_2H_5 & & C_3H_7\ (n) \\
| & & | \\
HC\text{—}CH_3 & \longrightarrow & HC\text{—}CH_3 \\
| & & | \\
CH_2COOH & & CH_2COOH \\
\\
\text{Dextro} & & \text{Levo} \\
\downarrow & & \downarrow \\
C_2H_5 & & C_3H_7\ (n) \\
| & & | \\
HC\text{—}CH_3 & & HC\text{—}CH_3 \\
| & & | \\
C_3H_7\ (n) & & C_2H_5 \\
\\
\text{Dextro} & & \text{Levo} \\
\\
\text{I} & & \text{II}
\end{array}
$$
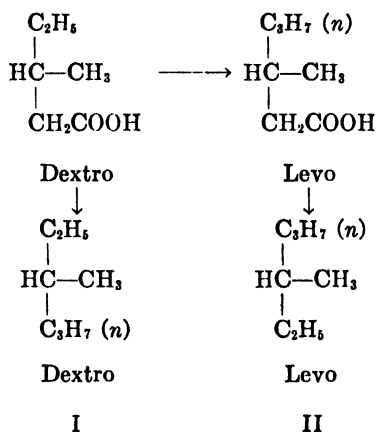
Thus one is confronted with the same question as in the case of secondary carbinols; namely, whether the configurationally related members of the methyl and of the ethyl series rotate in the same direction.

Should the general rule connecting the direction of rotation of the secondary carbinol with the structure be applicable to the hydrocarbons, then it is to be expected that all the members of the ethylmethyl series should remain dextrorotatory and that in the propylethyl series the members following the symmetric one should be dextrorotatory.   Furthermore, it is to be expected that in the butylethyl series, the first two members should be levorotatory and the third symmetric and the one to the right of the symmetric should be dextrorotatory.

Hydrocarbons of three series were prepared by sets of reactions indicated in Table I.   The rotations are summarized in Table II.

TABLE I

Experimental Values

$[M]_D^{25}$ (*in Degrees*

	—CHOH	—CH₂Br	—CH₂—COOH	—CH₂—CHOH—CH₃	—CH₂—CHOH—C₂H₅	—CH₂—CHOH—C₃H₇(n)	—C₃H₇(n)	—C₄H₉(n)	—C₅H₁₁(n)
CH₃	−2.13	+3.26	+4.25						
HC—									
C₂H₅	−1.97	+2.94		+4.25	+3.87	+5.28	+3.67	+5.07	+6.75

	—CH₂COOH	—CH₂—CH₂Br	—CH₂—CH₂—CHOH—CH₃	—CH₂—CH₃—CHOH—C₂H₅	—C₂H₅	—C₃H₇(n)	—C₄H₉(n)	—C₅H₁₁(n)
CH₃	+2.84*	−16.81*			+7.75			
HC—								
C₃H₇(n)	+1.28	−7.14	−0.46	−0.95		0	−0.59	−0.84
CH₃	+1.83	−5.44			+3.44		−0.57	−0.65
HC—								
C₄H₉(n)	+5.45*	−15.19*				+1.33		

* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

From Table II it can be seen that all members of the first series remain dextrorotatory.   This was to be expected as in the entire

TABLE II

Calculated Maximum Rotations of Configurationally Related Hydrocarbons

From experimental values on the basis of C_2H_5 having a $[M]_D^{25} = +10.35°$.

$$
\begin{array}{c}
HC-CH_3 \\
| \\
CH_2COOH
\end{array}
$$

$$
\begin{array}{c}
C_2H_5 \\
| \\
HC-CH_3 \\
| \\
CH_2COOH
\end{array}
$$

$+10.35°$

C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5
$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$
CH_2OH	CH_2Br	C_2H_5	$C_3H_7\ (n)$	$C_4H_9\ (n)$	$C_5H_{11}\ (n)$
$-5.18°$	$+7.91°$	0	$+9.87°$	$+13.63°$	$+18.16°$
$C_3H_7\ (n)$	$C_3H_7\ (n)$	$C_3H_7\ (n)$	$C_3H_7\ (n)$	$C_3H_7\ (n)$	$C_3H_7\ (n)$
$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$
CH_2COOH	CH_2CH_2Br	C_2H_5	$C_3H_7\ (n)$	$C_4H_9\ (n)$	$C_5H_{11}\ (n)$
$-3.60°$	$+20.06°$	$-9.87°$	0	$+1.67°$	$+2.36°$
$C_4H_9\ (n)$	$C_4H_9\ (n)$	$C_4H_9\ (n)$	$C_4H_9\ (n)$	$C_4H_9\ (n)$	$C_4H_9\ (n)$
$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$
CH_2COOH	CH_2CH_2Br	C_2H_5	$C_3H_7\ (n)$	$C_4H_9\ (n)$	$C_5H_{11}\ (n)$
$-7.26°$	$+20.20°$	$-13.63°$	$-1.81°$	0	$+0.86°$
$C_5H_{11}\ (n)$	$C_5H_{11}\ (n)$	$C_5H_{11}\ (n)$	$C_5H_{11}\ (n)$	$C_5H_{11}\ (n)$	$C_5H_{11}\ (n)$
$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$	$HC-CH_3$
CH_2COOH	CH_2CH_2Br	C_2H_5	$C_3H_7\ (n)$	$C_4H_9\ (n)$	$C_5H_{11}\ (n)$
$-11.84°$*	$+21.61°$	$-18.16°$	$(-2.36°)$†	$(-0.86°)$†	0

* Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

† Not experimental values but those of the enantiomorphous hydrocarbons.

series the heaviest radicle remains in the same position. In the other series the members to the left of the symmetric one are levorotatory. In these the upper radicle is heavier; the members to the right of the symmetric one are dextrorotatory inasmuch as in them the lower radicle is the heavier. Thus the direction of rotation of hydrocarbons seems to be determined by the respective allocation of the heavier group as was the case with the secondary carbinols.

Numerical Values—Several hydrocarbons had been previously prepared from active amyl iodide by the Wurtz method of synthesis. Thus, Marckwald[1] prepared methylethylpropylmethane, Welt[2] prepared methylethylpropyl-, methylethylbutyl-, methylethylamyl-, and methylethylisoamylmethane. The molecular rotations calculated from the recorded specific rotations were practically identical for all the members of this homologous series. *A priori*, this finding did not seem very probable. Indeed, from the results reported in this communication, it may be concluded that the substances previously prepared were impure. From Table II it can be seen that in every homologous series the values of the molecular rotation increase progressively. According to the rule of Tschugaeff, a maximum value should be reached. As yet our series have not been carried far enough to enable us to say when such a maximum is reached in the case of hydrocarbons.

It must be stated that the values given in Table II are calculated values. Inasmuch as all the reactions are accomplished without racemization it was possible to compute the values of the rotation of all substances on the basis of the maximum rotation of the 1,1-methylethylpropionic acid (3). It is interesting to note that the numerical values of the rotation of the two enantiomorphous methylpropylbutylmethanes, prepared on one hand from 1,1-methylpropylpropionic acid (3) and on the other from 1,1-methylbutylpropionic acids (3), are practically the same, thus indicating that the method of calculation employed was reliable.

Referring again to the numerical values of the rotations of the hydrocarbons given in Table II, it can be seen that those of the second row and in the third column are much higher than those given in the other rows and columns. This point will be made the subject of a separate discussion.

[1] Marckwald, W., *Ber. chem. Ges.*, **13**, 37, 1046 (1880).
[2] Welt, I., *Compt. rend. Acad.*, **119**, 745 (1894).

SUMMARY

1. The hydrocarbons of several homologous series have been prepared.

2. The rule which has been formulated by Levene and Haller in regard to the direction of the rotation of the secondary carbinols is applicable also to optically active hydrocarbons.

3. The maximum values of the rotation of the hydrocarbons have been calculated indirectly. Evidence is furnished to show that the method of calculation is reliable.

EXPERIMENTAL

Levo-2-Methyl Butanol-1—The inactive carbinol was resolved by recrystallizing the brucine salt of its half phthalic ester from acetone. After ten crystallizations the rotation of the phthalate was

$$[\alpha]_D^{23} = \frac{+ \ 0.25° \times 100}{1 \times 21.7} = + \ 1.15° \ \text{(in absolute alcohol)}$$

The brucine salt was decomposed with dilute hydrochloric acid and the phthalate hydrolyzed by boiling with potassium hydroxide. The carbinol was then distilled. B.p. 127° at 760 mm.; D $\frac{23}{4}$ = 0.816.

$$[\alpha]_D^{23} = \frac{- \ 1.97°}{1 \times 0.816} = - \ 2.41°. \quad [M]_D^{23} = - \ 2.12° \ \text{(homogeneous)}$$

Dextro-1-Bromo-2-Methyl Butane—50 gm. of 2-methyl butanol-1, $[\alpha]_D^{23} = -2.41°$, were cooled in ice and 100 gm. of phosphorus tribromide were slowly added. The product was let stand overnight at room temperature, then heated on a steam bath for 10 minutes. It was poured on ice and the oily layer separated. This was shaken with cold concentrated sulfuric acid then washed with dilute sodium carbonate solution, dried, and distilled. B.p. 119° at 760 mm.; D $\frac{22}{4}$ = 1.218.

$$[\alpha]_D^{23} = \frac{+ \ 2.63°}{1 \times 1.218} = + \ 2.16°. \quad [M]_D^{23} = + \ 3.26° \ \text{(homogeneous)}$$

The rotation obtained for this bromide is considerably higher than that previously reported in the literature. However, if the

directions for preparation, which are reported in the literature, are followed a much lower rotation is obtained due to racemization.

Dextro-2-Ethylbutyric Acid (4)—A Grignard reagent was prepared from 6 gm. of magnesium in dry ether and 35 gm. of 1-bromo-2-methyl butane, $[\alpha]_D^2 = +2.16°$. A stream of dry carbon dioxide was passed through the reagent for about 15 minutes. This Grignard solution was decomposed in the usual manner. The ether was evaporated and the residue dissolved in sodium hydroxide solution and extracted with ether. The alkaline solution was acidified and the organic acid extracted with ether and distilled. B.p. 110° at 30 mm.; yield 27 gm.; D $\frac{22}{4}$ = 0.925.

$$[\alpha]_D^{22} = \frac{+3.39°}{1 \times 0.925} = +3.66°. \quad [M]_D^{22} = +4.25° \text{ (homogeneous)}$$

3.460 mg. substance: 7.937 mg. CO_2 and 3.249 mg. H_2O.
$C_6H_{12}O_2$. Calculated. C 62.01, H 10.42
Found. " 62.55, " 10.50

Dextro-3-Methyl Hexanol-5—A Grignard reagent was prepared from 12 gm. of magnesium turnings in 500 cc. of dry ether and 77 gm. of 1-bromo-2-methyl butane, $[\alpha]_D^{22} = +1.95°$ (from 2-methyl butanol, $[\alpha]_D^2 = -2.24°$). The solution was cooled in ice and 30 gm. of acetaldehyde slowly run in with stirring. The Grignard solution was decomposed in the usual manner and the carbinol distilled. B.p. 146–147° at 760 mm.; yield 42 gm.; D $\frac{22}{4}$ = 0.816.

$$[\alpha]_D^{22} \quad \frac{+3.46°}{1 \times 0.816} = +4.24°. \quad [M]_D^{22} = +4.92° \text{ (homogeneous)}$$

3.114 mg. substance: 8.260 mg. CO_2 and 3.870 mg. H_2O.
$C_7H_{16}O$. Calculated. C 72.33, H 13.89
· Found. " 72.33, " 13.90

Dextro-3-Methyl Hexane—12 gm. of 3-methyl hexanol-5, $[\alpha]_D^{22} = +4.24°$, were distilled twice from 100 gm. of saturated hydriodic acid solution. The iodide was not isolated. This was added to an excess of zinc turnings and reduced by stirring and dropping in concentrated hydrochloric acid. After reduction was complete the hydrocarbon was extracted with ether and the ether distilled. The residue was purified by shaking with cold concentrated sulfuric acid followed by sodium carbonate solution. It was then

dried with anhydrous sodium sulfate and distilled from a small piece of sodium. B.p. 91–92° at 760 mm.; yield 5 gm.; D $\frac{22}{4}$ = 0.687.

$$[\alpha]_D^{22} = \frac{+\,2.52°}{1 \times 0.687} = +\,3.67°. \quad [M]_D^{22} = +\,3.67° \text{ (homogeneous)}$$

Calculated maximum rotation: $[\alpha]_D^{22} = +9.87°$. $[M]_D^{22} = +9.87°$
(homogeneous)
3.614 mg. substance: 11.145 mg. CO_2 and 5.170 mg. H_2O.
C_7H_{16}. Calculated. C 83.89, H 16.11
Found. " 84.09, " 15.99

Dextro-3-Methyl Heptanol-5—This carbinol was prepared from 8 gm. of magnesium in dry ether, 53 gm. of 1-bromo-2-methyl butane, $[\alpha]_D^{22} = +1.95°$, and 25 gm. of propionaldehyde, as described for 3-methyl hexanol-5. B.p. 72° at 22 mm.; yield 32 gm.; D $\frac{23}{4}$ = 0.816.

$$[\alpha]_D^{22} = \frac{+\,2.43°}{1 \times 0.816} = +\,2.98°. \quad [M]_D^{22} = +\,3.88° \text{ (homogeneous)}$$

2.760 mg. substance: 7.480 mg. CO_2 and 3.510 mg. H_2O.
$C_8H_{18}O$. Calculated. C 73.76, H 13.94
Found. " 73.90, " 14.23

Dextro-3-Methyl-5-Bromoheptane—30 gm. of 3-methyl heptanol-5, $[\alpha]_D^{23} = +2.98°$, were cooled in ice and treated with 60 gm. of phosphorus tribromide. This carbinol was very hard to brominate and was heated 2 hours on a steam bath. The bromide was purified as described for 1-bromo-2-methyl butane. B.p. 62° at 16 mm.; D $\frac{22}{4}$ = 1.077.

$$[\alpha]_D^{22} = \frac{+\,5.19°}{1 \times 1.077} = +\,4.82°. \quad [M]_D^{22} = +\,9.30° \text{ (homogeneous)}$$

4.675 mg. substance: 8.559 mg. CO_2 and 3.825 mg. H_2O.
$C_8H_{17}Br$. Calculated. C 49.73, H 8.88
Found. " 49.98, " 9.15

Dextro-3-Methyl Heptane—A Grignard reagent was prepared from 5 gm. of powdered magnesium in ether and 20 gm. of 3-methyl-5-bromoheptane, $[\alpha]_D^{22} = +4.82°$. The Grignard reagent was stirred and warmed for 15 minutes after final addition of the

halide.   It was then poured on ice and hydrochloric acid and the hydrocarbon extracted with ether.   The ether was evaporated and the hydrocarbon purified as described for 3-methyl hexane. B.p. 116–118° at 760 mm.; D $\frac{22}{4}$ = 0.710.

$$[\alpha]_D^{22} = \frac{+3.16°}{1 \times 0.710} = +4.45°. \quad [M]_D^{22} = +5.08° \text{ (homogeneous)}$$

Calculated maximum rotation: $[\alpha]_D^{22} = +11.97°$. $[M]_D^{22} = +13.63°$
(homogeneous)
2.705 mg. substance: 8.373 mg. CO_2 and 3.780 mg. H_2O.
C_8H_{18}.   Calculated.   C 84.10, H 15.90
Found.   " 84.13, " 15.87

Dextro-3-Methyl Octanol-5—This carbinol was prepared from 8 gm. of magnesium in dry ether, 53 gm. of 2-methyl-1-bromo-butane, $[\alpha]_D^{22} = +1.95°$, and 30 gm. of *n*-butylaldehyde as described for 3-methyl hexanol-5.   B.p. 89° at 15 mm.; yield 43 gm.; D $\frac{23}{4}$ = 0.822.

$$[\alpha]_D^{23} = \frac{+3.02°}{1 \times 0.822} = +3.67°. \quad [M]_D^{23} = +5.30° \text{ (homogeneous)}$$

3.526 mg. substance: 9.708 mg. CO_2 and 4.340 mg. H_2O.
$C_9H_{20}O$.   Calculated.   C 74.91, H 13.98
Found.   " 75.08, " 13.77

Dextro-3-Methyl-5-Bromooctane—43 gm. of 3-methyl octanol-5, $[\alpha]_D^{23} = +3.67°$, were cooled in ice and 60 gm. of phosphorus tribromide were added.   The product was heated $\frac{1}{2}$ hour on a steam bath, then poured on ice.   The halide was purified as described for 1-bromo-2-methyl butane.   B.p. 94° at 20 mm.; yield 47 gm.; D $\frac{23}{4}$ = 1.054.

$$[\alpha]_D^{23} = \frac{+5.93°}{1 \times 1.054} = +5.63°. \quad [M]_D^{22} = +11.65°$$

4.608 mg. substance: 8.862 mg. CO_2 and 3.810 mg. H_2O.
$C_9H_{19}Br$.   Calculated.   C 52.16, H 9.25
Found.   " 52.44, " 9.25

Dextro-3-Methyl Octane—A Grignard reagent was prepared from 6 gm. of magnesium in ether and 45 gm. of 3-methyl-5-bromo-octane, $[\alpha]_D^{23} = +5.63°$.   This was poured on ice and the hydro-

carbon extracted and purified as described for 3-methyl heptane. B.p. 143–144° at 760 mm.; yield 9 gm. (after purification); D $\frac{23}{4}$ = 0.725.

$$[\alpha]_D^{23} = \frac{+\ 3.82°}{1 \times 0.725} = +\ 5.27°. \quad [M]_D^{23} = +\ 6.75° \text{ (homogeneous)}$$

Calculated maximum rotation $[\alpha]_D^{23} = +14.18°$. $[M]_D^{23} = +18.16°$
(homogeneous)

3.675 mg. substance: 11.371 mg. CO_2 and 5.185 mg. H_2O.

C_9H_{20}. Calculated. C 84.27, H 15.73
Found.   " 84.37, " 15.79

Levo-5-Methyl-2-Octanol—A Grignard reagent was prepared from 8 gm. of magnesium in 200 cc. of dry ether and 60 gm. of 1-bromo-3-methyl hexane, $[\alpha]_D^{21} = -3.99°$. To this reagent were added 15 gm. of acetaldehyde in ether. The Grignard solution was decomposed and the carbinol obtained in the usual manner. B.p. 92° at 15 mm.; yield 35 gm.; D $\frac{25}{4}$ = 0.821.

$$[\alpha]_D^{25} = \frac{-\ 0.52°}{2 \times 0.821} = -\ 0.32°. \quad [M]_D^{25} = -\ 0.46° \text{ (homogeneous)}$$

3.955 mg. substance: 10.870 mg. CO_2 and 4.875 mg. H_2O.

$C_9H_{20}O$. Calculated. C 74.91, H 13.98
Found.   " 74.94, " 13.79

Levo-5-Methyl Octane—35 gm. of 5-methyl-2-octanol, $[\alpha]_D^5 = -0.32°$, were distilled twice from 200 gm. of hydriodic acid, sp. gr. 1.70. The crude iodide was not purified. This was placed in a 1 liter flask with 200 gm. of zinc turnings and reduced by adding slowy 500 cc. of concentrated hydrochloric acid. It was placed on a steam bath when the initial reaction subsided, until the zinc was dissolved. The hydrocarbon was extracted with ether and the ether distilled. The crude hydrocarbon was shaken with cold concentrated sulfuric acid, washed with sodium carbonate solution, then water, and dried with dry sodium sulfate. It was refluxed 1 hour with a small piece of metallic sodium and then fractionated.   B.p. 53° at 25 mm.; yield 22 gm.; D $\frac{22}{4}$ = 0.714.

$$[\alpha]_D^{22} = \frac{-\ 0.65°}{2 \times 0.714} = -\ 0.46°. \quad [M]_D^{22} = -\ 0.58° \text{ (homogeneous)}$$

3.491 mg. substance: 10.785 mg. CO_2 and 4.956 mg. H_2O.
C_9H_{20}. Calculated.   C 84.27, H 15.73
　　　　Found.　　　" 84.24, " 15.88

Levo-6-Methyl-3-Nonanol—This carbinol was prepared by the action of propionaldehyde on a Grignard reagent formed from 8 gm. of magnesium in ether and 60 gm. of 1-bromo-3-methyl hexane, $[\alpha]_D^{24} = -3.99°$. B.p. 105° at 15 mm.; yield 29 gm.; D $\frac{24}{4}$ = 0.820.

$$[\alpha]_D^{24} \quad \frac{-0.53°}{1 \times 0.820} = -0.65°. \quad [M]_D^{24} = -1.02° \text{ (homogeneous)}$$

5.010 mg. substance: 13.855 mg. CO_2 and 6.260 mg. H_2O.
$C_{10}H_{22}O$. Calculated.   C 75.86, H 14.02
　　　　Found.　　　" 75.41, " 13.98

Levo-6-Methyl Nonane—29 gm. of 6-methyl-3-nonanol, $[\alpha]_D^{24} = -0.65°$, were distilled twice with 200 gm. of hydriodic acid, sp. gr. 1.70. The iodide was separated and reduced by zinc and hydrochloric acid as described for·5-methyl octane. B.p. 72° at 25 mm.; yield 8 gm.; D $\frac{22}{4}$ = 0.731.

$$[\alpha]_D^{22} = \frac{-0.86°}{2 \times 0.731} = -0.59°. \quad [M]_D^{22} = -0.84° \text{ (homogeneous)}$$

4.485 mg. substance: 13.880 mg. CO_2 and 6.240 mg. H_2O.
$C_{10}H_{22}$. Calculated.   C 84.40, H 15.60
　　　　Found.　　　" 84.39, " 15.57

Levo-6-Methyl-3-Decanol—This carbinol was prepared by the action of 25 ,gm. of propionaldehyde on the Grignard reagent formed from 12 gm. of magnesium in ether and 80 gm. of 1-bromo-3-methyl heptane, $[\alpha]_D^{24} = -2.79°$. The carbinol was isolated in the usual way. B.p. 117° at 22 mm.; yield 35 gm.; D $\frac{24}{4}$ = 0.829.

$$[\alpha]_D^{24} = \frac{-0.54°}{2 \times 0.829} = -0.33°. \quad [M]_D^{24} = -0.56° \text{ (homogeneous)}$$

4.990 mg. substance: 14.045 mg. CO_2 and 6.085 mg. H_2O.
$C_{11}H_{24}O$. Calculated.   C 76.66, H 14.05
　　　　Found.　　　" 76.75, " 13.65

Levo-6-Methyl Decane—30 gm. of 6-methyl-3-decanol, $[\alpha]_D^{24} = -0.33°$, were heated with 200 gm. of hydriodic acid, sp. gr. 1.70,

until the iodide was formed.   The oily layer as separated and re-
duced by zinc and concentrated hydrochloric acid as described for
5-methyl octane.   B.p. 94° at 30 mm.; yield 8 gm.; $D \frac{24}{4} = 0.738$.

$$[\alpha]_D^{24} = \frac{-0.58°}{2 \times 0.738} = -0.39°. \quad [M]_D^{24} = -0.61° \text{ (homogeneous)}$$

3.410 mg. substance: 10.541 mg. CO_2 and 4.820 mg. H_2O.
$C_{11}H_{24}$.   Calculated.   C 84.51, H 15.49
Found.   " 84.29, " 15.81

Dextro-3-Methyl Heptane—A Grignard reagent was prepared
from 6 gm. of magnesium in ether and 50 gm. of 1-bromo-3-methyl
heptane, $[\alpha]_D^{24} = -2.79°$.   The Grignard reagent was poured on
ice and the hydrocarbon was isolated and purified as described
for 5-methyl octane.   B.p. 117–118° at 760 mm.; yield 14 gm.;
$D \frac{22}{4} = 0.710$.

$$[\alpha]_D^{22} \frac{+2.24°}{1 \times 0.710} +3.15°. \quad [M]_D^{22} = +3.60°$$

3.852 mg. substance: 11.865 mg. CO_2 and 5.465 mg. H_2O.
C_8H_{18}.   Calculated.   C 84.10, H 15.90
Found.   " 84.00, " 15.87

THE COMPARATIVE ANTIRACHITIC AND CALCIFYING PROPERTIES OF IRRADIATED MILK AND MILK DERIVATIVES

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PLATES 1 AND 2

Although the synthesis of vitamin D by the action of ultra-violet rays upon ergosterol seems to have been fairly well established, numerous researches (1–11) have indicated many factors which must be taken into consideration in order to assure preparation of the antirachitic principle with uniform potency and constant composition. While the majority of investigations have had as their object the determination of optimum conditions for the production of irradiated ergosterol as a therapeutic agent, laboratory and clinical studies have frequently directed attention to the activatability of various foods, more particularly milk.

The published data from which definite conclusions may be drawn in regard to the maximum degree of antirachitic potency attainable by milk or its derivatives are meager. The investigations of Hess and Weinstock (12) of Steenbock *et al.* (13, 14), and of Supplee and Dow (15) have shown that milk and butter fat can be activated to a substantial degree. Numerous clinical data (16–23) have also shown the favorable results from the use of irradiated milk, and it is significant that such data are remarkably free from reports of adverse results, although the favorable response has not always been as rapid as from the use of the more established therapeutic agents.

In view of the variability in reported potency of different ergosterol preparations, it may be assumed that complete standardization of the steps of preparation has not yet been attained, and that uniform preparations are not readily duplicated in the same or different laboratories. While there is not generally available an abundance of data regarding the optimum conditions for the activation of milk, it would seem that if the optimum conditions for the treatment of this product or any of its derivatives were ascertained and established, a product having relatively uniform properties could be readily produced and duplicated. It is probable that the inherent composition of milk fixes, within narrow limits, the amount of ergosterol or provitamin which may be potentially activated, and it is also probable that in milk the provitamin bears a fairly constant physical and chemical relationship to the other constituents of the milk. · Such a relationship would tend to limit variations in the receptivity to radiant energy of a given quantity and quality. It may be assumed that one of the factors which determines the degree to which milk may be activated is the amount of provitamin which it contains. No direct evidence indicating the ergosterol content of milk or its derivatives has been found in the literature. In view of the potentialities contingent upon knowledge concerning the activation of milk, one of the general objectives of the work reported in this paper was to obtain, if possible, an estimation of the ergosterol or provitamin content of milk; a further objective was a comparative study of the activatability of certain derivatives of milk.

Preliminary Work with Non-Saponifiable Fraction of Milk Fat

Our initial efforts were centered upon an attempt to obtain from the non-saponifiable fraction of milk fat a sterol concentrate which could be activated to a degree at least commensurate with that already known to be attainable by irradiation of the natural product. Of the various procedures which seemed to be best suited for the purpose, the general method of Windaus and Grosskopf (24) seemed preferable and was followed.

2 kilos of roller process dry whole milk were extracted with 3 liters of 95 per cent alcohol for about 4 weeks, or until no more color was removed by the solvent. Approximately 540 gm. of alcohol-soluble matter were recovered. This was saponified with

a mixture consisting of 400 cc. of aqueous KOH (2:1) and 1000 cc. of alcohol for 3 hours in a water bath.   It was rapidly filtered through a Buchner filter and distilled to one-half its volume or approximately 800 cc.   An equal volume of water was then added, followed by 2 liters of ethyl ether.   This mixture was thoroughly shaken in a separatory funnel, the ether layer removed, and the extraction again repeated with the same volume of ether.   The ether extracts were combined and rapidly washed with 500 cc. of dilute HCl (1:9); the ether extract was then dried with anhydrous sodium sulfate.   Upon evaporation of the solvent a bright yellow oil was obtained which solidified into a waxy mass on cooling.   This residue had a distinct honey-like odor.

This non-saponifiable residue was taken up with the minimum amount of petroleum ether (about 5 cc.).   On subsequent cooling in a freezing mixture a white crystalline precipitate was thrown out.   This was filtered off and washed in a cold pack funnel maintained as nearly as possible at the crystallization temperature. This crystalline precipitate was designated as "crude crystals" and the petroleum ether-soluble part designated as the "yellow oil" fraction.   The crude crystals weighed 917 mg.   The weight of the yellow oil fraction was not obtained at this juncture.   The crude crystals were repeatedly redissolved in boiling petroleum ether and reprecipitated by cooling in a freezing mixture. This procedure was carried out about fifteen times.   On each occasion a quantitity of white crystals was obtained from which it became increasingly more difficult to remove the solvent because of the formation of a substance which appeared as floating oil drops relatively insoluble in warm petroleum ether.   This substance assumed the character of a dirty white, pasty mass on cooling. An attempt was made to remove it from the mixture of precipitated crystals and petroleum ether-soluble portion, which latter continued to yield a yellow oil fraction as in the first precipitation. This relatively insoluble pasty substance was designated as "residual oil."   Each increment appearing on successive resolution of the white crystals was saved, yielding a total of 684 mg. after evaporating the petroleum ether at room temperature in a 15 inch vacuum.   The combined petroleum ether portions soluble at 0°, or yellow oil fraction, yielded 1542 mg. after evaporation of the solvent in a vacuum at low temperature.   The crystalline

residue, now desginated as the "white crystal" fraction, weighed 212 mg.

It was noted that after the first precipitation the crystalline substance readily turned yellow on exposure to air and light.   This apparent instability however became less pronounced on successive recrystallizations.   The question therefore naturally arises whether the residual oil which appeared during the process of purification may not have been a decomposition product, rather than a substance originally present in the non-saponifiable residue. The unsaturated and unstable character of the higher sterols suggests the former possibility.

Each of the fractions obtained from the above procedure was prepared for testing as follows: The solvent-free substances were dissolved in alcohol in the ratio of 25 mg. per 61 cc. and the solutions divided into three parts; one portion was irradiated for 16 seconds and another for 5 minutes by a Hanovia quartz mercury vapor lamp with reflector burning at 145 volts and 4.5 amperes. The depth of the alcoholic solution was approximately 2 mm. and the distance from the lamp was 12 inches.   The third portion was not irradiated.   Immediately after irradiation the alcohol was evaporated and the residues taken up with a predetermined amount of pure olive oil.   From this stock oil solution subsequent dilutions were made with calculated amounts of the same oil so that each 0.2 cc. contained the amount of test substance which it was desired to feed each test animal per day.   The non-irradiated portions were similarly diluted with olive oil.

For determining the antirachitic and calcifying properties of the various fractions, white rats 28 to 30 days old were selected from our standardized stock colony.   The test animals were placed on the Steenbock rickets-producing diet, Ration 2965,[1] for a period of 21 days, followed by a 10 day feeding period of the test substance.   At the end of this period the microscopic line test was applied and bone ash determinations made according to our usual procedure.   From Table I it will be noted that the results from these fractions were of a negative character.

Numerous unpublished data obtained at this Laboratory have shown that fluid milk containing substantially 1.2 per cent butter

[1] Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).

fat, irradiated for 16 seconds under controlled conditions and fed at a 10 cc. level for 10 days, brings about an appreciable degree of healing (++ to +++) in rachitic rats subjected to the same testing and diagnostic technique. These results are also usually

TABLE I

Results of Feeding Irradiated Non-Saponifiable Fractions of Milk Fat for 10 Days to Rachitic Rats

Test substance	Period of irradiation	Amount fed per day	Bone ash, average per cent	Line test
	sec.	*mg.*		
Ration 2965			29.24	— —
White crystals	None	0.0025	33.76	— —
	16	0.0025	32.62	— —
	16	0.0625	33.16	— —
	16	0.3125	31.97	— —
	16	2.0000	32.11	— —
	300	2.0000	32.41	— —
	300	20.0000	33.35	— (?)
Yellow oil	None	0.0025	31.87	— —
	16	0.0025	32.32	— —
	16	0.0625	33.85	— —
	16	0.3125	31.39	— —
	16	2.0000	29.82	— —
	300	2.0000	32.41	— *
	300	20.0000	28.82	— —
Residual oil	300	20.0000	27.90	— —
White crystals and yellow oil	16 16	0.3125 0.3125	30.29	— —
White crystals, yellow oil, residual oil	16 · 16 16	0.3125 0.3125 0.3125	29.26	— —*

* Hemorrhagic condition in bone shaft.

accompanied by an increase of 3 to 5 per cent in bone ash as compared with control animals receiving the same amount of non-irradiated milk. On the basis of the yield of the non-saponifiable increments obtained from the milk fat, an equivalent of the white crystal fraction ingested with the milk fed at the level mentioned

above would be about 0.0514 mg. per day, the yellow oil fraction about 0.342 mg. per day, and the residual oil fraction about 0.1512 mg. per day. These figures compared with the feeding levels recorded in Table I show the lack of calcifying power of the test fractions as compared with a similar and smaller intake of non-saponifiable milk fat substance irradiated and ingested as a component of the milk. The question of too low feeding levels of the prepared fractions can be eliminated as an explanation of the negative results. In the light of the fragmentary data available, imperfect methods for obtaining the provitamin in a form that can be activated are at once suggested. However, the obvious conclusion which may be drawn from these results is that the preparation of antirachiticly active non-saponifiable fractions from natural substances which it is known can be activated is not simple.

Not only is the negative character but also the possibility of an anticalcifying effect of the non-crystalline fractions a matter to be considered, particularly when these fractions are irradiated for the longer periods of time and fed at the higher levels. On the other hand the results from the white crystal fraction indicate a trend toward positive calcification even though concurrent healing of rickets was not manifested.

Use of Chickens for Study of Antirachitic Potency of Irradiated Non-Saponifiable Fraction of Milk Fat

The results from the preliminary work with the irradiated non-saponifiable fraction of milk fat prompted further investigations involving a somewhat different technique. Only the total non-saponifiable fraction of the fat was used for these studies. Chickens were used for test purposes as they appeared to offer the possibility of a greater refinement in interpretation of results if the preventive method of testing were used (25). The work of other investigators (26), reported after these studies were started, indicates that the results from rats and chickens may not be comparable.

Day old White Leghorn chicks, hatched from a standard flock maintained on a standardized ration which was designed to produce uniform stock for experimental purposes, were obtained from Cornell University. A basal ration particularly adapted for our purpose because of the liberal percentage of skim milk solids

was used, the composition of which follows: skim milk powder 20 parts, flour wheat middlings 20 parts, steamed bone meal 2 parts, yellow corn-meal (whole corn) 57.5 parts, and sodium chloride 0.5 part. Groups of twenty to thirty chickens were housed in pens of suitable area and brooder facilities in a room from which all outdoor light was excluded. Each pen was lighted 14 hours per day with one 40 watt frosted Mazda lamp. Weekly food consumption records were kept for all groups, and in certain instances daily records were maintained. All chicks were weighed and carefully examined each week. At the termination of the test period six or eight individuals from each group were selected for postmortem diagnosis, including blood phosphorus and calcium analyses, radiographic examination, macroscopic line test of the sectioned bone, bone ash, and observations of the ribs and costochondral junctions. Selection of the specimens for postmortem examination was made by taking an equal number of males and females nearest the average weight of the particular group.

Negative and positive control groups were included in the series. One positive control group contained 1 per cent standard grade cod liver oil as prepared for human consumption by a well known manufacturer. The cod liver oil was freshly mixed with the basal ration once a week. Additional control groups were given viosterol. Both the viosterol and cod liver oil were obtained direct from the same manufacturer. In order that the amount of viosterol fed might be uniformly maintained at a comparable level, the daily food consumption of the cod liver oil group was determined and the average cod liver oil intake per chick calculated. An amount of viosterol was provided equivalent to the cod liver oil intake. The viosterol was diluted in proper proportions with olive oil and this mixture fed by pipette in such quantities that the total amount of oil received per chick per day was the same as that ingested by those receiving cod liver oil.

The test substances used for this series of experiments consisted of the total non-saponifiable fraction of milk fat. No attempt was made to fractionate this residue as was done in the preliminary tests with rats. Dry whole milk and a good grade of commercial butter were used for obtaining the test fractions. Those fractions irradiated were dissolved in ether at a 3.5 per cent concentration; the ether solution was dropped on quartz plates and spread over

an area about 9 square inches during evaporation of the solvent. The amount of non-saponifiable matter in each film thus prepared was approximately 20 mg. The films were exposed to the total emanations of the quartz mercury vapor lamp with reflector at a distance of 12 inches for 16 seconds or for 5 minutes. The irradiated material was washed from the plates with ether, the various increments combined, and the solvent evaporated. The treated fractions were then taken up with a predetermined amount of olive oil from which suitable dilutions with more oil were subsequently
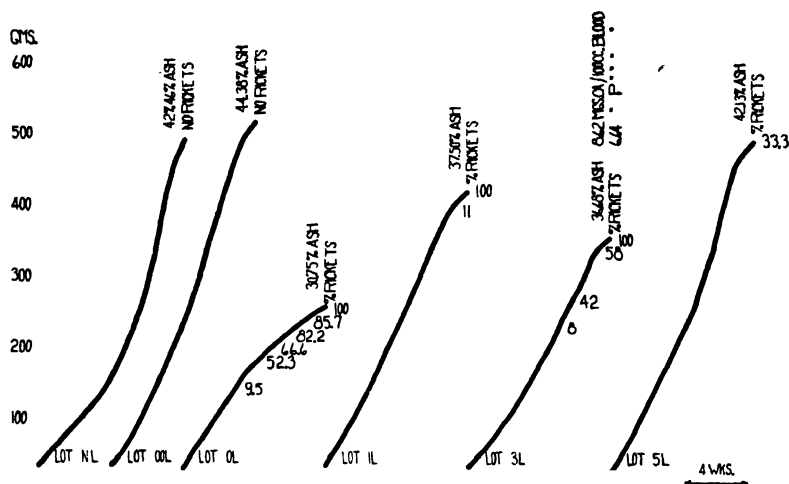
CHART I. Growth and incidence of rickets in chickens under various test conditions. Lot NL, normal complete ration. Lot OOL, 1 per cent cod liver oil, 245.86 mg. average per day. Lot OL, basal ration only. Lot IL, viosterol, 2.45 mg. average per day. Lot 3L, viosterol, 2.39 mg. average per day. Lot 5L, viosterol, 4.90 mg. average per day.

made for feeding as already described for the viosterol groups. Some groups were fed arbitrarily the test substances at 0.01 the amount of the cod liver oil intake, others at a 0.001 level, and still other groups were fed an amount equivalent to the yield of non-saponifiable substance from a definite quantity of milk fat; the amount of fat used as the basis of calculation was determined by quantities ingested in experiments described hereinafter (Chart IV). In addition to the test fractions, certain groups received a supplement of non-irradiated butter oil fed at graduated

levels predetermined by the amount of butter fat ingested from the rations above indicated. The yield of non-saponifiable residue was as a rule 0.4 to 0.6 per cent of the fat. The purpose of the butter oil supplement was to ascertain whether the natural milk fat carried a complementing factor which would affect the results.
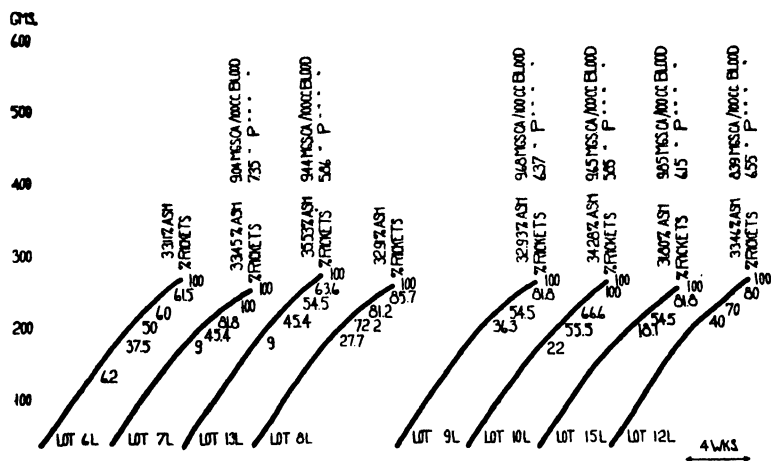
CHART II. Growth and incidence of rickets in chickens fed the non-saponifiable fraction of milk fat, irradiated and non-irradiated, with and without butter oil supplements. Lot 6L, non-saponifiable fraction of milk fat non-irradiated, 0.245 mg. average per day. Lot 7L, non-saponifiable fraction of milk fat irradiated 16 seconds, 0.245 mg. average per day. Lot 13L, non-saponifiable fraction of milk fat irradiated 16 seconds, 0.245 mg. average per day, and non-irradiated butter oil, 552.3 mg. average per day. Lot 8L, non-saponifiable fraction of milk fat irradiated 5 minutes, 0.245 mg. average per day. Lot 9L, non-saponifiable fraction of milk fat non-irradiated, 2.45 mg. average per day. Lot 10L, non-saponifiable fraction of milk fat irradiated 16 seconds, 2.45 mg. average per day. Lot 15L non-saponifiable fraction of milk fat irradiated 16 seconds, 2.45 mg. average per day, and non-irradiated butter oil, 552.3 mg. average per day. Lot 12L, non-saponifiable fraction of milk fat irradiated 5 minutes, 2.45 mg. average per day.

Viosterol was also supplemented with butter oil and with small graduated amounts of the non-irradiated non-saponifiable residue of milk fat.

The results of this series of tests are shown in Charts I to III in which incidence of rickets, both clinical and postmortem,

growth rate, bone ash, and, for certain lots, blood calcium and phosphorus are recorded. In these and in subsequent charts the percentage of rickets indicated at the end of the observation
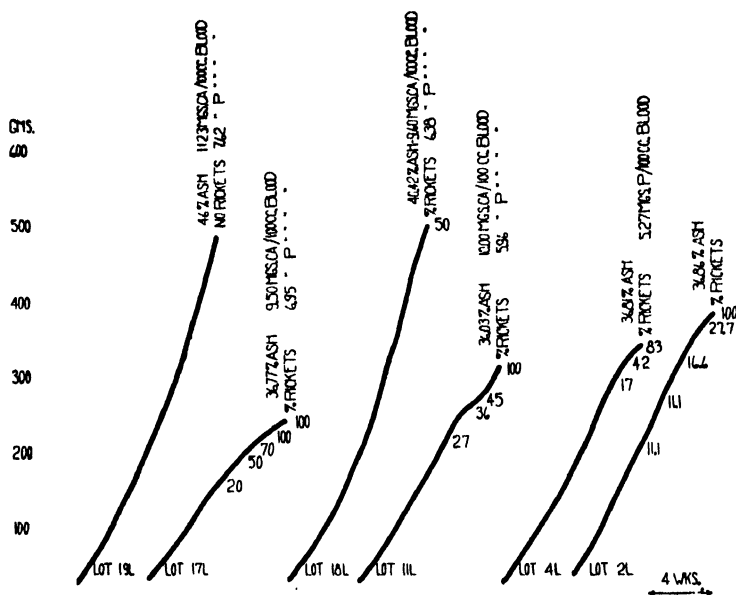
CHART III. Comparative effect on growth and incidence of rickets in chickens fed irradiated butter oil and its irradiated non-saponifiable fraction, as well as the effect of a similarly irradiated fraction of milk fat. Lot 19L, butter oil irradiated 16 seconds, 520.5 mg. average per day. Lot 17L, non-saponifiable fraction of butter oil non-irradiated, 4.00 mg. average per day (equivalent to 520 mg. of butter oil). Lot 18L, non-saponifiable fraction of butter oil irradiated 16 seconds, 3.23 mg. average per day (equivalent to 520 mg. of butter oil). Lot 11L, non-saponifiable fraction of milk fat irradiated 16 seconds, 2.57 mg. average per day (equivalent to 520 mg. of butter oil). Lot 4L, viosterol, 2.45 mg. average per day, and non-irradiated butter oil, 648.48 mg. average per day. Lot 2L, viosterol, 2.45 mg. average per day, and non-saponifiable fraction of milk fat non-irradiated, 0.245 mg. average per day.

period is based upon postmortem examination, whereas the percentage of rickets previous to the final examination is based upon clinical diagnosis only.

Comparative Antirachitic Value of Irradiated Milk and Milk Derivatives Determined by Use of Chickens

Further tests of the antirachitic value of irradiated milk and milk products irradiated for varying periods of time were made for the purpose of comparing their respective merits for the prevention of rickets in chickens. Skimmed milk, a partially skimmed milk containing 0.5 per cent fat, a milk containing 1.2 per cent fat, and milk containing 3.4 per cent fat were irradiated in fluid form under uniform conditions of exposure and energy output by quartz mercury vapor lamps of the Hanovia type with suitable reflectors, for periods varying from 16 to 208 seconds. The lamps had been previously used for approximately 400 burning hours. The irradiated milk was subsequently dried by the double roller process. A fat-casein-albumin-free, water-soluble milk vitamin concentrate dissolved in water at a 12 per cent concentration was also tested after irradiation in a thin film 2 mm. in depth for 32 seconds at a distance of 12 inches from the lamp. Butter oil was tested after irradiation on glass plates in a thin film not exceeding 0.5 mm. in thickness with an exposure period of 16 seconds at a distance of 12 inches from the lamp.

Suitable adjustments were made when the irradiated dry milk replaced the dry skimmed milk of the basal ration; the percentage of corn-meal was so altered that all rations were maintained at the same protein level. 5 per cent of the solid matter of the water-soluble milk vitamin concentrate was incorporated in the basal ration with slight adjustment of the corn-meal to equalize the protein content. The groups receiving the butter oil were fed daily by pipette the same amount of the oil as was ingested by the group receiving the milk powder which had been irradiated for 16 seconds. The chickens receiving the skimmed milk powders, the 5 per cent fat milk powders, and the water-soluble vitamin concentrate were Barred Plymouth Rocks, whereas all others in this and in preceding series were White Leghorns. Due to the difference in the rate of growth of the two breeds, it was necessary to reestablish negative and positive control records for the heavier breed. 1 per cent cod liver oil of the same grade as was used in the preceding series served as one of the positive controls.

A second positive control consisted of the basal ration only, and direct irradiation of the chickens for 10 minutes per day six times per week at a distance of 26 inches from the mercury vapor lamp. The observation period for all groups in which the Barred

FIG. 1. Lot 10L (Chart II) received non-saponifiable fraction of milk fat irradiated 16 seconds. 2.45 mg. (equivalent to 552 mg. of fat) average received per day. 100 per cent rickets at 7 weeks.

FIG. 2. Lot 22L (Chart IV) received dry milk containing 12 per cent fat irradiated 48 seconds. 670.7 mg. of fat average intake per day. No rickets at 8 weeks.

Plymouth Rocks were used was 10 weeks instead of 8. Otherwise, and with the exceptions noted, the technique was the same as for the preceding series. The results from these groups are shown in Charts IV to VI. Figs. 1 and 2 are included to show the typical condition of average specimens of certain lots from the two series of tests. Figs. 3 and 4 show the presence or absence of rickets in

certain of the experimental lots as revealed by bone sections stained with silver nitrate.

The relative antirachitic potency of certain of these irradiated products was also determined by the standard curative technique with rats wherein 1 gm. of dry substance per day was fed for a period of 10 days following a period of 21 days on rachitic Ration
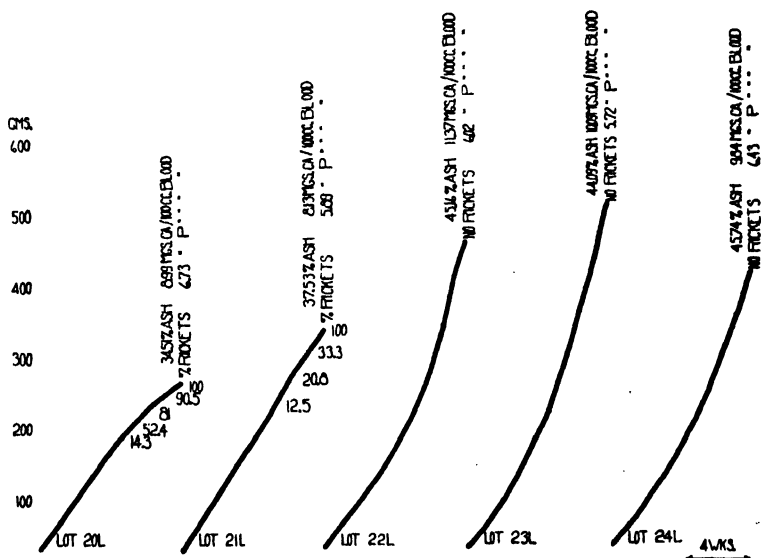
CHART IV. Growth and incidence of rickets in chickens fed dry milk of variable fat content irradiated for variable periods of time. Lot 20L, dry milk, 12 per cent fat, non irradiated, 401.28 mg. average fat per day. Lot 21L, dry milk, 12 per cent fat, irradiated 16 seconds, 520.05 mg. average fat per day. Lot 22L, dry milk, 12 per cent fat, irradiated 48 seconds, 670.70 mg. average fat per day. Lot 23L, dry milk, 12 per cent fat, irradiated 96 seconds, 587.25 mg. average fat per day. Lot 24L, dry milk, 27.5 per cent fat, irradiated 16 seconds, 1721.23 mg. average fat per day.

2965. The skimmed milk irradiated for 16 seconds showed a negative line test; after 48 seconds irradiation a + line was consistently shown; no greater degree of healing resulted after 208 seconds exposure. Bone ash results were not consistently higher than those shown by the animals receiving the non-irradiated skimmed milk. The milk powder containing 5 per cent butter fat and irradiated for 16 seconds consistently showed a + degree of

healing; irradiation for 48 seconds as a general rule showed a ++ degree of calcification. The water-soluble milk vitamin concentrate irradiated for 32 seconds consistently gave a + result. Consistent increase in bone. ash was not shown for this product even though measurable calcifying properties were indicated by the line test. This has been a no♥ infrequent occurrence. The dry milk samples containing higher amounts of fat have shown at
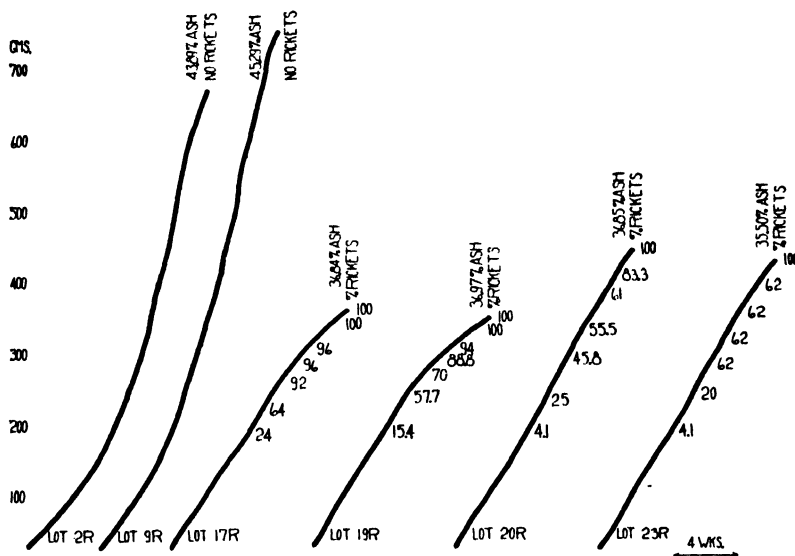
CHART V. Growth and incidence of rickets in chickens fed skimmed milk powder irradiated for variable periods of time. Lot 2R, ultra-violet light. Lot 9R, 1 per cent cod liver oil. Lot 17R, skimmed milk powder non-irradiated (basal ration only). Lot 19R, skimmed milk powder irradiated 16 seconds. Lot 20R, skimmed milk powder irradiated 48 seconds. Lot 23R, skimmed milk powder irradiated 208 seconds.

least a +++ degree of calcification after irradiation for 16 seconds or more. This degree of healing is also accompanied by an increase of from 3 to 5 per cent bone ash greater than that shown by animals receiving the same quantity of non-irradiated milk during the 10 days test period. ·

SUMMARY

Negative antirachitic and calcifying properties were obtained from the irradiated non-saponifiable fraction of milk fat, as

determined by the curative method with white rats and the preventive method with chickens.

Milk irradiated in fluid form and subsequently dried by the double roller process showed marked antirachitic properties. The degree of potency which may be imparted to milk irradiated in fluid form is, with limitations, determined by the fat content and period of exposure, other things being equal. An irradiation
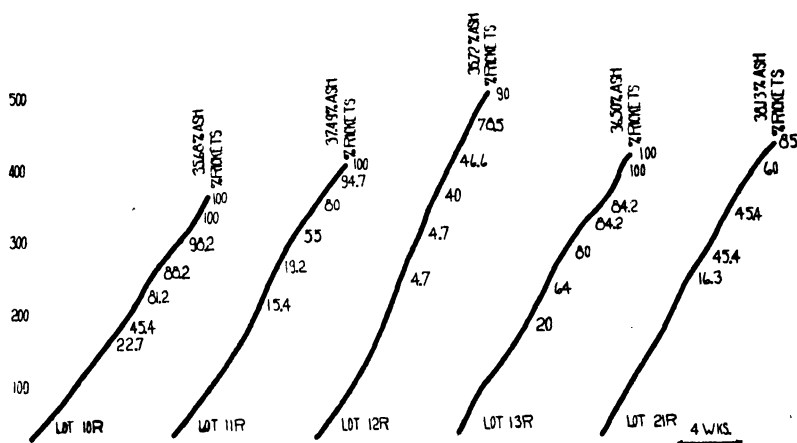
CHART VI. Growth and incidence of rickets in chickens receiving a fat-free milk derivative and milk powder containing 5 per cent fat, irradiated for variable periods of time. Lot 10R, dry milk, 5 per cent fat, non-irradiated. Lot 11R, dry milk, 5 per cent fat, irradiated 16 seconds. Lot 12R, dry milk, 5 per cent fat, irradiated 48 seconds. Lot 13R, skimmed milk powder non-irradiated (basal ration), and 5 per cent water-soluble milk vitamin concentrate non-irradiated. Lot 21R, skimmed milk powder non-irradiated (basal ration), and 5 per cent water-soluble milk vitamin concentrate irradiated 32 seconds.

period of only a few seconds is sufficient, under suitable conditions, to activate a particular milk sample to a high percentage of its potential maximum. This degree of activation is attained without detectable change in taste or odor.

The negative results obtained from the irradiated non-saponifiable fractions of milk fat as contrasted with the positive character of the results obtained from irradiation of the natural milk,

butter fat, and a fat-casein-albumin-free, water-soluble fraction of milk, provide further evidence of the difficulties encountered in the preparation of antirachitic concentrates from known activatible natural products.

The use of chickens and the basal ration containing a uniform amount of milk solids supplying a natural balance of milk constituents provides a method of particular merit for the study of the antirachitic and calcifying properties of activated milk or milk derivatives. Any attempted explanation of the failure of the irradiated non-saponifiable fractions to show the anticipated antirachitic activity must, therefore, center around speculative possibilities attending the manipulations of preparation. Oxidation of the provitamin prior to extraction might be suggested by the fact that the non-saponifiable fraction of butter oil gave relatively better results than similar fractions obtained from the fat extracted from milk powder. The relative difference in these results however does not entirely exclude the possibility that the solvent failed to free the provitamin from the non-fatty constituents of the milk powder. These possibilities considered in the light of the markedly favorable results obtained by irradiation of the natural products present an interesting field for speculation regarding the physicochemical relationship of the provitamin to the other milk constituents.

The failure of viosterol to give the same degree of protection to chickens as cod liver oil, when fed at twice the recommended level and standardized against the same cod liver oil according to the rat technique, presents further stimulation for study of the mechanism of the biological activity of natural products and the extracted and highly concentrated preparations.

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EXPLANATION OF PLATES 1 AND 2

FIGS. 3 AND 4. Calcification of the epiphyses of tibiæ as shown by sections stained with silver nitrate.
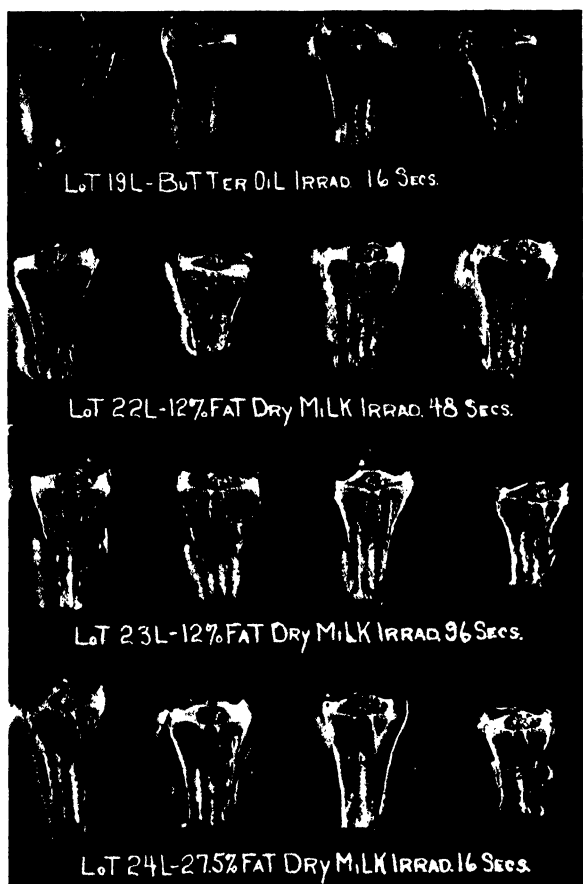
-

LoT 19L-BuTTER OiL IRRAD. 16 Secs.

LoT 22L-12%FAT DRy MiLK IRRAD. 48 Secs.

LoT 23L-12%FAT DRy MiLK IRRAD 96 Secs.

LoT 24L-27.5%FAT DRy MiLK IRRAD.16 Secs.

Fig. 3

Fig. 4

THEELIN. SOME PHYSICAL AND CHEMICAL PROPERTIES*

By SIDNEY A. THAYER, LOUIS LEVIN, and EDWARD A. DOISY

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In a recent paper (Veler, Thayer, and Doisy, 1930) we described a method of extraction and purification of theelin. Using this procedure with only minor modifications we have made many batches of the crystals and have gradually accumulated a supply which we hope will enable us to determine the structure of the compound. Since this aspect of our investigations will probably take some time we are publishing certain data on the physical and chemical properties that seem to be well established.

In a preliminary paper (Thayer, Veler, and Doisy, 1930) we have given data on the ultimate analysis, molecular weight, melting point, iodine number, and esterification of theelin. Shortly after the publication of that paper on the chemistry of theelin, Butenandt's (1930) study of the same subject appeared. Butenandt's data agreed with ours on the composition and molecular weight of theelin, but differed from ours on the nature of one oxygen linkage. Since our work on this point was incomplete due to an inadequacy of the supply of theelin, we have taken up the study again as soon as theelin became available. We have found in agreement with Butenandt that theelin contains one hydroxyl and one carbonyl group thereby accounting for both atoms of oxygen.

The discovery of theelol (Doisy, Thayer, Levin, and Curtis, 1930) has absorbed so much of our time, that the work on theelin has been delayed. Theelol exhibits both physiological and chemical similarities to theelin but we believe that the evidence submitted (Curtis and Doisy, 1931; Thayer, Levin, and Doisy, 1931) is sufficient to show that two different compounds have been isolated.

EXPERIMENTAL

Elementary Composition

Nitrogen[1] was determined by Pregl's micro-Dumas method on Preparation 119 c 42. The weight of the sample was 4.588 mg.; no nitrogen.

TABLE I

Elementary Composition of Theelin; Carbon and Hydrogen

Preparation No.	Weight of sample	CO_2	H_2O	C	H
	mg.	*mg.*	*mg.*	*per cent*	*per cent*
F 153 + F 156* (mixed)	2.940	8.595	2.245	79.72	8.54
F 153 + F 156* "	3.015	8.805	2.275	79.64	8.44
199	5.271	15.460	3.908	79.99	8.29
198	5.393	15.810	4.050	79.95	8.40
199 h	3.828	11.240	2.845	80.08	8.31
199 h	3.642	10.652	2.730	79.77	8.39
Average..				79.86	8.39

* By Research Service Laboratories, New York.

Molecular Weight

Our experience with the Rast micro method was gained with naphthalene, carbanilide, and cholesterol. Control determinations with cholesterol were carried out several times during the determination of the molecular weight of theelin. Our complete data indicate that the error of this method in our hands is less than 5 per cent.

The average of ten determinations on four different samples is 274. As we will show in the next section of this paper this value

[1] Analysis by Mr. C. D. Veler, to whom we express our thanks.

agrees with the molecular weight determined by indirect methods. Examination of the combustion data of Table I and the molecular weight determinations of Table II indicates the probable formula of $C_{18}H_{22}O_2$. Calculated: carbon, 79.95; hydrogen, 8.21; molecular weight, 270.

TABLE II

Molecular Weight of Theelin Determined by Rast's Method

Sample	Weight of sample	Weight of camphor	Δ°C.	Molecular weight
	mg.	*mg.*		
Preparation 164	0.240	3.202	11.3	267
	0.349	2.168	24	268
	0.388	2.130	27	269
Preparation A 160	0.145	2.666	8	272
	0.275	2.655	15	276
Cholesterol	0.349	1.804	20	387
Preparation F 153	0.643	6.236	14	295
	0.437	3.947	16	277
	0.223	2.004	16	278
Preparation F 156	0.314	3.124	15	268
	0.194	2.625	11	269
Cholesterol	0.836	5.483	16	381

$$M = \frac{40 \times \text{weight of sample} \times 1000}{\Delta \times \text{weight of camphor}}.$$

Iodine Number, Double Bonds, and Molecular Weight

Owing to the ease of oxidation, the determination of the iodine number is attended with some difficulty. It is well known that cholesterol uses more iodine than its one double bond accounts for. In earlier work on the follicular hormone Jordan and Ralls[2] studied the determination of the iodine number of cholesterol and devised a micro procedure that gave good results with both cholesterol and other unsaturated lipids. This method has been used by us for cholesterol and theelin.

[2] Unpublished work from this laboratory. Our thanks are also due Dr. C. N. Jordan for other assistance.

From the iodine numbers of three different preparations (Table III) of theelin an average of 95.1 is obtained. With this value, the presence of one double bond being assumed, the molecular weight can be calculated by a simple proportion. With this indirect procedure the calculated molecular weight is 266, which agrees fairly well with the result 274 obtained by the Rast method.

The iodobromotheelin was isolated by making the acetic acid solution faintly alkaline and then extracting with chloroform. The chloroform solution was washed with water, distilled, and the residue recrystallized from hot alcohol by the gradual addition of hot water. The compound was dried over $CaCl_2$ and P_2O_5. When we attempted to obtain the melting point, the compound

TABLE III

Iodine Number of Theelin

Sample		Weight	Iodine No.	
		mg.		
Cholesterol		6.800	67.2	
"		4.400	66.6	
"		3.800	67.8	
"		4.710	68.9	
"		5.249	62.4	Average 66.6
				Theory 65.7
Preparation 164		3.771	96.6	
"	F 153	4.455	92.8	
"	F 156	3.769	96.0	Average 95.1

turned dark brown and decomposed (203–215°). Physiological assay of this derivative dissolved in olive oil gave 350 rat units per mg. which is approximately one-tenth of the number for the original untreated sample.

Halogen was determined on 2.8 mg. by a combination of the Carius decomposition and the Whitehorn (1920–21) titration. Found 1.202 mg. or 42.92 per cent halogen. Calculated for the molecular weight of the iodobromo derivative 481; for theelin by subtraction of 206.8 (IBr) 274.

Oxygen and Hydroxyl Groups

Since our analysis of theelin showed that it contained carbon and hydrogen but no nitrogen and since Laqueur (Dingemanse, de

Jongh, Kober, and Laqueur, 1930) did not find halogens or phosphorus in his analysis of menformone, it was assumed that the discrepancy between 100 per cent and the sum of the percentages of carbon and hydrogen was due to oxygen. In our preliminary work we obtained data which indicated the presence of two hydroxyls in theelin, but this has not been confirmed by our more recent experiments. The improvised micro methods employed were not satisfactory from the standpoint of accuracy. As soon as sufficient theelin was available the method of Peterson and West (1927) was used for the quantitative determination of hydroxyl groups.

89.8 mg. of Preparation 199 e were dissolved in pyridine and acetic anhydride (3:1) and heated at 95° under a micro reflux

TABLE IV

Analysis of Monoacetyl Derivative of Theelin

Preparation No.	Weight of sample	CO_2	H_2O	C	H
	mg.	*mg.*	*mg.*	*per cent*	*per cent*
199 e	5.635	15.870	3.945	76.81	7.83
199 e	4.810	13.595	3.423	77.08	7.96
198	3.317	9.355	2.365	76.92	7.98
198	3.940	11.100	2.785	76.83	7.95
Theoretical 1(OH)...........................				76.88	7.75

condenser for 21 hours. The solution was transferred quantitatively to a 250 cc. flask containing ice-cold water and titrated with standard NaOH. The difference between the blank and the sample was 0.56 cc. of 0.5755 N NaOH giving a value of 41.67 gm. CH_3CO per mol of theelin. Gravimetric determination of the ester gave a value of 35.09 gm. CH_3CO per mol. Theoretical for $C_{18}H_{21}O(OH)$ is 43 gm. per mol. The compound formed by acetylation was isolated and recrystallized from alcohol by the addition of water (see Fig. 1). Several recrystallizations gave a pure white crystalline compound that melted sharply at 125.3° (uncorrected). The analysis of two specimens of the acetate (Table IV) gave values that agree well with the theoretical for monoacetyl theelin.

Hydrolysis of Acetate

27.8 mg. of ester (Preparation 199 e) were hydrolyzed by warming with dilute sodium hydroxide. The solution was acidified with hydrochloric acid, the precipitate filtered off and recrystallized from 25 per cent ethyl alcohol. Melting point 250.7° (uncorrected); bioassay 3000 rat units per mg. Analysis for carbon and hydrogen is given in Table I (Preparation 199 h). The crystalline appearance, melting point, bioassay, and analysis leave little doubt about the recovery of the original compound by hydrolysis of the ester.

Identification of Hydroxyl by Methylation

Theelin was methylated by shaking a sample dissolved in dilute sodium hydroxide with dimethyl sulfate. A precipitate formed shortly after starting this treatment. The mixture was finally warmed to complete the reaction, then cooled, and filtered. The solid was dissolved in ethyl alcohol and precipitated by the addition of dilute aqueous sodium hydroxide. It was recrystallized by dissolving in alcohol and precipitating with water. For a photograph of the crystals see Fig. 2. The crystals softened at 163° and melted at 165° (uncorrected).

Sample mg.	CO_2 mg.	H_2O mg.	C per cent	H per cent	Theory ($C_{19}H_{24}O_2$) C H	
2.784	8.170	2.150	80.04	8.64	80.24	8.51
3.225	9.460	2.470	80.00	8.57	80.24	8.51

Methoxyl

Sample mg.	AgI mg.	OCH_3 per cent	Theory ($C_{19}H_{24}O_2$)
2.713	2.225	10.83	10.92

Identification of Carbonyl Group

Since esterification gave only a monoacetyl derivative, we attempted to prepare an oxime. The first attempt in an alkaline solution was unsuccessful but heating theelin with hydroxylamine in ethyl alcohol acidified with acetic acid gave the oxime. The addition of water produced a precipitate which was recrystallized from 95 per cent ethyl alcohol and also from alcohol by the addi-

tion of hot water (see Fig. 3). The product melted with decomposition at 229° (uncorrected). Combustion was so difficult that lead chromate was mixed with the sample in the carbon and hydrogen and potassium chlorate in the nitrogen determinations.

Sample	CO_2	H_2O	C	H	Theory ($C_{18}H_{23}O_2N$)	
mg.	mg.	mg.	per cent	per cent	C	H
3.025	8.410	2.200	75.82	8.13	75.72	8.13
2.938	8.175	2.155	75.89	8.20	75.72	8.13

Nitrogen (Micro-Dumas Method)

Sample	Barometric pressure at 0°	Volume of N	N	Theory ($C_{18}H_{23}O_2N$)
mg.	mm.	cc.	per cent	
3.740	752.2	0.156	4.67	4.90

Fig. 1. The monoacetyl derivative of theelin; recrystallized from dilute ethyl alcohol. 80 ×. A photograph of theelin was published previously (Doisy, Veler, and Thayer, 1930).

Specific Rotation

The specific rotation was determined in a 2 dm. tube with the sodium flame and the sample dissolved in 95 per cent ethyl alcohol. Whether the values contained in Table V indicate a variation in the purity of our samples or are the result of technical errors we are unable to say. This work will be repeated under more favorable conditions when we obtain a larger quantity of theelin. It is interesting to note that of the isolated hormones, this is the first to show a dextrorotation.

Fig. 2. The monomethyl ether of theelin; recrystallized from dilute ethyl alcohol. 228 ×.

Fig. 3. The oxime of theelin; recrystallized from dilute ethyl alcohol. 228 ×.

TABLE V

Specific Rotation of Theelin

Preparation No.	Concentration	Observed rotation	Specific rotation
	per cent	*degrees*	*degrees*
H 1832	0.107	+0.36	+166
N 182	0.560	+1.75	+156*
F 174	0.073	+0.23	+157

' Micro tube used.

Melting Point

In our earlier papers we gave the melting point with decomposition of theelin at 243–243.5° (uncorrected). Actually this is the temperature at which a few minute, brown, oily droplets appear. The back-bone which is light gray in color does not melt until a temperature of 247–249° (uncorrected) is reached. Sufficient of one of our earlier preparations, Preparation A 160 (see Thayer, Veler, and Doisy, 1930), was left so that we could repeat its melting point. Similar to our later preparations it melted at 249°. Our failure to notice this behavior sooner was due to the fact that we were trying to ascertain whether the sample melted with decomposition as determined by bioassay. Minute droplets detected with a magnifying glass began to appear at 243°. Without heating more, the tube was rapidly withdrawn from the bath, cleaned on the outside, and crushed in 95 per cent ethyl alcohol. Preparation A 160, which originally assayed 4000 rat units per mg., showed a potency of only 1000 units after being heated at 244° in a sealed capillary. Our preparations have generally had melting points ranging from 247–249° (uncorrected) but in two instances the melting points were 250.7° and 250.2°. In the case of the sample melting at 250.7° the original theelin which melted at 249° had been esterified, then recrystallized, and finally hydrolyzed and recrystallized. Perhaps, the higher figure is correct and the slightly lower figures are due to traces of impurities that cannot be removed from theelin by repeated recrystallization. Preparation A 160 which had been recrystallized twenty times from several solvents, was snow-white yet melted at 249° (uncorrected).

The temperatures corrected for stem exposure are about 7° higher than the uncorrected value. Two sets of calibrated short stem thermometers have been used for certain samples. The melting point of Preparation 199 by this procedure was 256.2°; with the 360° Bureau of Standards thermometer 249° (uncorrected).

DISCUSSION

Our data lead to the belief that theelin is a compound having the formula $C_{18}H_{22}O_2$. One of the atoms of oxygen exists as a hydroxyl which is easily acetylated; the other as a carbonyl which readily reacts with hydroxylamine. One double bond which adds iodine

is present in the molecule. It is obvious from a consideration of the formula that theelin must contain other double bonds or a system of ring structures. In examining these possibilities Butenandt has prepared the compound $C_{18}H_{30}O$ by hydrogenation. Further work will be necessary to decide the nature of the ring structure in theelin.

The question of the solubility in dilute alkali has already been discussed by Butenandt (1929). In his earlier paper he gave $C_{23}H_{28}O_3$ as the probable formula and expressed the view that the solubility in alkali was due to a lactone linkage. In his later paper (1930) in which he gives the formula $C_{18}H_{22}O_2$ he attributes the solubility to enolization of the carbonyl. We believe that our evidence indicates that the solubility is due to acidic ionization of the hydroxyl.

The evidence upon which our opinion is based is not entirely convincing but nevertheless is suggestive. Both the ester and methyl ether of theelin are insoluble in dilute alkali. Furthermore, both theelin and theelol give a positive Millon's reaction but the ethers respond negatively to this test. Tentatively, we conclude that the solubility of theelin in dilute sodium hydroxide is due to a phenolic hydroxyl.

SUMMARY

1. Theelin is a keto monoatomic alcohol having a formula $C_{18}H_{21}O(OH)$.

2. Acetylation gives a monoacetyl derivative having a melting point of 125.3° (uncorrected). Theelin can be recovered by hydrolysis of the ester.

3. Methylation produces a monomethyl ether melting at 165° (uncorrected).

4. An oxime melting with decomposition at 229° (uncorrected) has been prepared.

5. Theelin contains one double bond that adds iodine. It melts at 254–257° (corrected); probably the higher temperature is correct for the pure compound. Theelin is dextrorotatory.

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